NOTES ON THE *HOW* TECHNIQUE FOR THE MICRO-ESTIMATION OF ARSENIC

by T. WILKINSON AND C. G. GREENHAM

(From the Division of Plant Industry, Commonwealth Council for Scientific and Industrial Research, Canberra).

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Many workers in biological fields are interested in the quantitative estimation of amounts of arsenic as low as 0·1γ. Recently How (1938) devised a modification of the Gutzeit method suitable for estimating such small amounts.

We have experienced considerable difficulty in establishing the technique, and the following notes are presented to help those who may wish to use this delicate but intricate method.

Apparatus, Etc.

The zinc alloy. To obtain the requisite rate of dissolution, we found it essential to use for the zinc alloy much more iron than is used by How. With our reagents[1] a suitable alloy was obtained by melting together the following: Zinc—99·5 gm.; tin—0·500 gm.; lead—0·010,0 gm.; iron—0·010,0 gm. The materials for the alloy (270 gm.) were melted in a 180 × 20 mm. silica test tube inserted into a vertically arranged unit of an electric combustion furnace. We cast single blocks by pipetting the molten alloy into moulds of asbestos cement, in preference to following How's procedure of cutting up a long cylinder. On the average, 28 blocks were obtained from the above quantity.

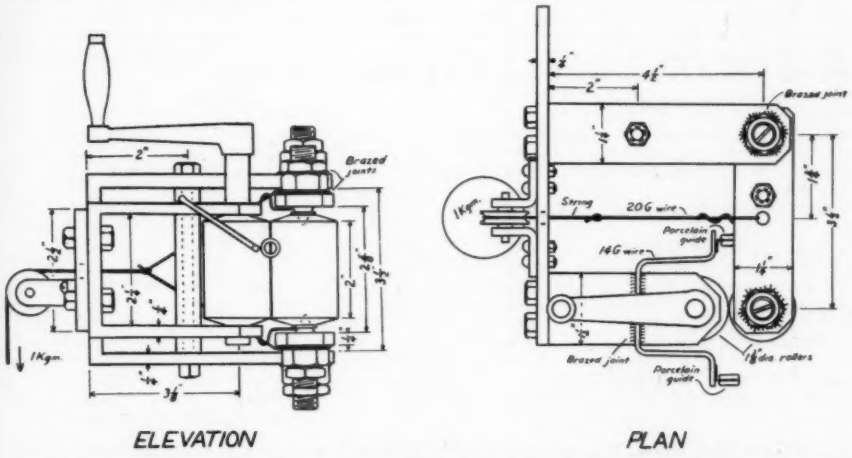

Fig. 1. *Constructional details of wringer.* The two rollers were made of insulation fibre; probably ebonite would be better. The outer roller is fitted with a brass bush. The arm carrying the outer roller is pivoted on cone bearings similar to those used for the outer roller. This arm is rendered rigid laterally by two cross bars; the position of one of these cross bars is indicated by the hexagonal nut on the arm. The other cross bar is drilled to take the cone bearings. In use, the outer roller is wiped in such a fashion that no extra force is applied to the impregnated string.

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[1] Zinc (Baker's analysed, batch No. 112, 537): As—0·000,000 p.c., Pb—0·005 p.c., Fe—0·003 p.c.; Iron (Baker's analysed, powder): As—0·000 p.c.; Tin (Merck's granulated): As—0·000,05 p.c.; Lead ("Analar"): As_2O_3—0·000,1 p.c.

The generation tubes. It was necessary to purchase a gross of 23 × 150 mm. Pyrex test tubes in order to select 24 which were fairly uniform. We found it advisable to pair the tubes and baffles. Due to technical difficulties we were obliged to use 43 ml. of solution, as against 40 ml. used by How.

The absorption units. The capillary tubing for these units was supplied as being approximately 1·30 mm. diameter. Using the mercury thread technique, 26 pieces 6 cm. in length, and varying in diameter from 1·32 to 1·38 mm., were obtained. How recommends tubing 1·3 mm. in diameter. With our tubing it was necessary to substitute a slightly thicker crochet cotton (Dolfuss-Maeg et Cie. Retors à Broder, No. 4) for the Morse and Kaley knitting cotton.

In constructing the water scrubbers it was necessary to make the glass caps of such a length that they could not be forced up out of the water by the hydrogen.

The Standardization of the Technique.

Using 0·25 p.c. alcoholic mercuric chloride solution, and following How's technique as closely as possible, in no instance did we obtain lengths of stain comparable with those obtained by him. Moreover, there was no reproducibility among replicates, and the stains were frequently faint.

Longer stains, without better reproducibility, were obtained by reducing the concentration of mercuric chloride to 0·125 p.c. This concentration was used when obtaining the results described below.

Although our alloy contained tin and iron as recommended, the reproducibility of replicates and the quality of stains were somewhat improved by the following procedure: 2 ml. of 5 p.c. Mohr's salt (slightly acidified with sulphuric acid) were pipetted into each generation tube; after transferring the digested material and adding the sodium bisulphite in solution, and diluting, 0·2 ml. of 40 p.c. stannous chloride solution in concentrated hydrochloric acid was added.

It was also found that the length of stain was largely dependent on the pressure with which the impregnated string was wrung. In order to obtain constant results, a wringer was required in which the pressure could be easily adjusted and reproduced, with the surface of the rollers such that the string would take all the pressure (Fig. 1). The removal of excess fluid from the roller faces was also found to be necessary. It was immaterial whether the string was dried in the vertical or horizontal position. String which was 2–3 days old did not give consistent results; therefore, freshly impregnated string was invariably used.

Table 1 shows the reproducibility of the stain lengths for 1γ of arsenic. The determinations were carried out on different days, with absorption units selected at random.[2] Table 2 shows a typical set of stain lengths obtained for a range of standards. All our standards were put through the digestion process, which alters the length of stain obtained. Standards should be run for each batch of zinc alloy until the procedure is shown to be unnecessary.

TABLE 1.

	Arsenic (in γ)	Stain Length (in mm.)
Zinc 7	1·0	9·5
		9·5
		9·0
		9·0
Zinc 5	1·0	9·0
		9·5
		9·0
		9·0
Zinc 7	1·0	9·5
		9·0
		9·5

TABLE 2.

	Arsenic (in γ)	Stain Length (in mm.)
Zinc 2	1·0	1·5
		1·5
		1·5
	0·5	4·5
		5·5
		5·0
	0·9	7·5
		7·5
		7·5
	1·3	11·5
		9·5
		11·0
	1·7	13·5
		14·0

Acknowledgements. We are indebted to A. E. How, Esq., for a gift of some Morse & Kaley knitting cotton, received through the courtesy of the Australian Trade Commissioner's Office, New York. We also wish to thank Mr. G. A. McIntyre, at present of this Division, for his valuable criticism and help in statistical interpretations.

REFERENCE.

How, A. E. (1938): Ind. Eng. Chem. Anal. Ed., 10, p. 226.

[2] The acids used were: Sulphuric acid (Baker's analysed), As—0·000,000 p.c.; Nitric acid (Baker's analysed), As—0·000,000,1 p.c.; Perchloric acid (Baker's analysed), 60 p.c. vacuum distilled.

THE ESTIMATION OF COBALT IN PLANT AND ANIMAL TISSUES

by HEDLEY R. MARSTON AND DOUGLAS W. DEWEY

(From the Nutrition Laboratory of the Council for Scientific and Industrial Research, Division of Animal Health and Nutrition, University of Adelaide, South Australia).

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Subsequent to the recognition that cobalt plays a fundamental part in the aetiology of Coast Disease, which affects sheep grazing on the calcareous littoral of Southern Australia, the general importance of cobalt in ruminant nutrition has been widely appreciated. The literature recently has been reviewed briefly (Marston, 1939).

Although circumstantial evidence strongly suggests that cobalt is an essential dietary constituent, at least for ruminants, final proof must await a more direct means of approach, and this will depend on the precise estimation of the minute amounts of cobalt involved.

This investigation was undertaken to overcome the disabilities in existing analytical procedures.

The interaction between cobalt and naphthoquinone–(1·2) oxime–(1), (α nitroso β naphthol), in dilute acetic acid to form a bulky insoluble precipitate which was described by Ilinski and Knorre (1885), has been extensively used for separating nickel and cobalt. In 1885 Hoffmann prepared the α nitroso compound of Schaeffer's acid, 2–oxynaphthalin–sulphonic acid–(6), and reported its interaction with cobalt and with iron to form respectively a brilliant red and an intense green dyestuff. More recently, v. Klooster (1921) prepared the α nitroso derivative of R–acid, 2–oxynaphthalin–disulphonic acid (3·6), and suggested that this compound might prove useful for the detection of small quantities of cobalt as, in faintly acid or neutral solutions, it forms a soluble red cobalt complex which persists in hot and relatively strong nitric acid, under which conditions similar complexes with other metals are decomposed. Stare and Elvehjem (1932) demonstrated that the red colour produced under these conditions is proportional to the amount of cobalt in the reacting solution, providing the reagent is in excess. They cautiously used the reaction for the colorimetric estimation of quantities of cobalt down to 0·05 mg. Other investigators, Kidson, Askew and Dixon (1936), and McNaught (1939), have introduced minor modifications in the v. Klooster reaction and have claimed a high degree of sensitivity.

Colorimetric estimations based on this reaction have hitherto lacked precision owing to interference by the yellow colour of the excess reagent, which persists with only slight modification after boiling in nitric acid. Expedients used by the above workers who match the final colour produced with a series of similarly prepared standards in Nessler tubes, have only partially overcome the difficulties involved. Moreover, the conditions under which the reaction proceeds to completion have not been appreciated fully and the influence of other reacting substances has not been studied exhaustively.

The reaction of naphthoquinone–(1·2)–oxime–(1) disulphonic acid (3·6), with nitric acid and with bromine in acid solution. The deep yellow naphthoquinone–(1·2)–oxime–(1) disulphonic acid(3·6), (Nitroso R. Salt), is converted to the light yellow (1) nitro–(2) oxynaphthalin disulphonic acid–(3·6), with the evolution of nitrous fumes when boiled with nitric acid of moderate strength, the reaction being similar to that observed by Stenhouse and Groves (1887), to occur when α nitroso β naphthol is treated similarly. When the nitro compound in 2N nitric acid reacts at 70° C. with excess of bromine, four molecules of bromine are consumed and a colourless product results after removal of excess bromine by boiling. This might imply direct addition of four bromine atoms in the remaining unsubstituted (4, 5, 7, 8) positions of the naphthalin nucleus. It was found that considerably less bromine will discharge the colour; the addition of very little more than one molecular equivalent of bromine to a solution of nitroso R. salt, previously boiled in nitric acid, will result in a colourless solution, and so substitution in the reactive (4) position is apparently completed prior to bromination proceeding further.

Bromination, under these conditions, provided a simple means of discharging the colour of the excess reagent.

The nature of the coloured complex formed by the interaction of cobalt with naphthoquinone (1·2)–oxime (1) disulphonic acid–(3·6) and its reaction with nitric acid and with bromine. Alkaline conditions favour a shift of the equilibrium towards the oxime form which reacts readily with cobalt, the soluble co-ordination compound, $(C_{10}H_4NO_2.H_2S_2O_6)_3Co$, being produced. This is very highly coloured and extraordinarily stable. It may be boiled in citrate solutions at pH 8·0 without any decomposition and quantitatively withstands boiling in 4N. nitric acid for at least fifteen minutes. It remains unchanged in 2N. nitric acid for twenty-four hours at room temperature if guarded from intense light. At reactions between pH 5·0 and 8·0, its formation proceeds rapidly to remarkable completion. *In relatively strong acid solution it is unaffected by bromine.*

Fig. 1 was derived from experiments in which quantities of nitroso R. salt ranging from 0·5 to 5·0 molecular equivalents reacted at room temperature for five minutes with 1 ml. of 0·001 M. $Co(NO_3)_2$ in 20 ml. of approximately ·04 M. phosphate: citrate buffer at pH 7·0. The intensity of colour after the acidity was increased to 2N. with HNO_3, the solution boiled, and the volume adjusted to 25 ml., was found to be directly proportional to the amount of N.R.S. in the reacting mixture up to a limit of three equivalents of the reagent. To this point, no excess of the yellow reagent remained; the reaction was complete. Excess of N.R.S. over this limit was unchanged and after boiling with nitric acid, the yellow colour of the corresponding nitro compound was observed to persist in quantity sufficient to render impossible direct colorimetric comparison. This residual colour was found to be discharged completely by adding an excess of bromine to the warm solution and removing that remaining after a few minutes by boiling, a procedure which proved to exert no effect on the intensity of the colour of the red cobalt–N.R.S. complex.

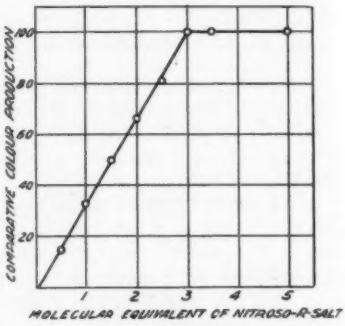

Fig. 1. The stoicheiometric relationship between Co. and Nitroso R. salt.

Under these conditions, the complex withstands the action of at least thirty times the quantity of bromine necessary to discharge completely the residual colour of the total amount of the N.R.S. reagent employed in the reaction. The action of bromine on the preformed Co–N.R.S. complex in acid solution was investigated further under a large variety of conditions and observed to exert no effect. This wide tolerance towards bromine under conditions which totally discharge the colour of the residual reagent renders it possible to adapt the procedure for direct quantitative colorimetry.

The intensity of the Co–N.R.S. complex and the Lambert-Beer Law relationships. When the colour due to excess of reagent was removed, the intensity of the red cobalt complex was found to be directly proportional to the concentration of cobalt in the solution, and even at very high dilutions, it was found possible to estimate the intensity by direct colorimetry. In solutions containing the Co–N.R.S. complex from 1 γ of cobalt in 10 ml., for example, the colour intensity of a 30 mm. depth of solution may be estimated with considerable precision. Two series of ten determinations of the colour in this concentration had standard errors of $\pm$ 0·265 mm. and $\pm$ 0·183 mm. respectively, when estimated by two observers, and the mean of each series was within $\pm$ 5 p.c. of the theoretical value. In solutions containing 2 γ Co in 10 ml., ten estimations of the colour match between layers 30 mm. in depth had a S.E. of $\pm$ 0·104 mm. and $\pm$ 0·175 mm. respectively, the means of each series being with $\pm$ 2 p.c. of the theoretical reading. The colour of solution containing 2 γ in 10 ml. was matched with one containing 5 γ in 10 ml. at 30 mm. depth with a S.E. of $\pm$ 0·058 mm. and $\pm$ 0·069 mm. by individual observers and the means of each ten observations were within $\pm$ 1 p.c. of theory. At concentrations exceeding 5 γ Co in 10 ml. the colour is very easily matched within the usual $\pm$ 1 p.c. limits of direct colorimetry.

Extension of the observations proved the molecular extinction coefficient to be remarkably constant over a very wide range of cobalt concentrations.

The influence of reaction on the formation of the Co–N.R.S. complex. The influence of reaction on the rate and completeness of the formation of the Co–N.R.S. complex was investigated over a range extending from pH 2·0 to pH 10·0 in the presence of a variety of buffer salts. For this purpose, 50 γ of cobalt in 8 ml. of solution containing 2 ml. of the particular buffer, standardized to $\pm$ 0·01 pH, were treated with 0·2 ml. of 1·0 p.c. solution of nitroso R. salt. After exactly five minutes, 4 ml. of 10N. nitric acid were added, and the solution boiled for one minute. The solution was then cooled to approximately 70° C., 0·5 ml. of 0·2N. bromine solution added, and bromination allowed to proceed for five minutes. Excess of bromine was then removed by boiling, the solution cooled, made up to 20 ml. and the intensity of colour estimated against appropriate standards.

The findings are expressed in Fig. 2. At acidities greater than pH 2·5 no complex is formed. Less acid conditions favour its formation and the interaction reaches completion in five minutes at room temperature at about pH 6·0. Curves relating pH of the system with relative velocity of interaction between cobalt and N.R.S. as measured by the amount of coloured complex produced in five minutes at room temperature, were sigmoid in shape. They were readily reproducable and characteristic of the type of buffer employed. In the range extending from pH 6·0 to pH 8·0, the formation of the cobalt N.R.S. complex proceeds rapidly to completion in all of the buffers studied. More alkaline reactions tend to precipitate cobaltous hydroxide and so complicate the formation of the complex by the introduction of a solid phase; but the safe pH range may be extended on the alkaline side by the addition of citrate. The formation of the complex is not quantitative in glycine buffers between pH 8·0 and pH 10·0, and the presence of ammonia in this range seriously influences the production of the Co–N.R.S. complex, presumably, owing to the formation of other cobalt complexes with the ammonia and the amino acid.

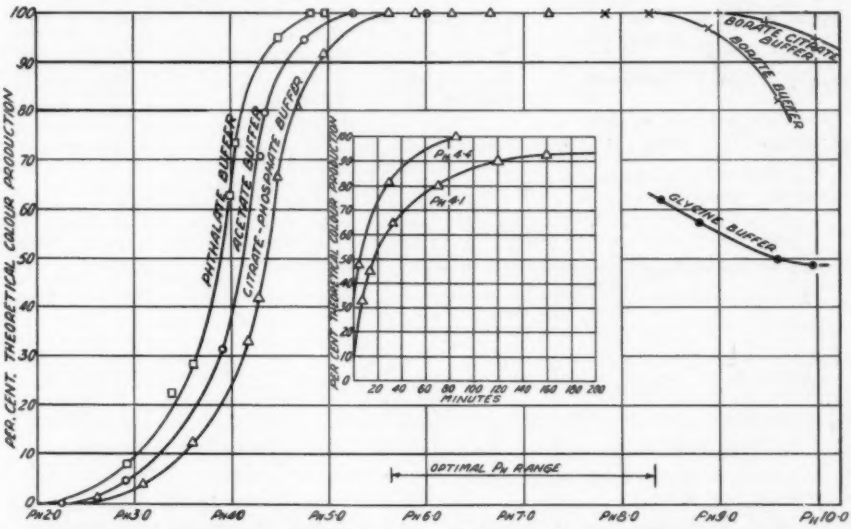

Fig. 2. The influence of reaction on the completeness of the formation of Co–N.R.S. in three minutes at room temperature.

(Inset) The influence of reaction on the rate of the formation of Co–N.R.S.

The temperature co-efficient of the interaction is large and the rate of formation of the coloured complex is enhanced considerably by warming the solution. Heating in the more alkaline range in the absence of citrates favours the irreversible formation of cobaltic oxide and thus should be avoided. The inset in Fig. 2 illustrates the influence of reaction on the velocity of the formation of the complex at room temperature.

The influence of other ions on the formation of the Co–N.R.S. complex. The interaction between cobalt and N.R.S. is uninfluenced by high concentrations of $'Cl$, $''SO_4$, $'NO_3$, $'''PO_4$, acetate or citrate ions; thus, the complex was formed rapidly and completely between pH 6·0 and pH 8·0 in the presence of NaCl, $Na_2SO_4.10H_2O$, $NaNO_3$, Na acetate and Na citrate in concentrations corresponding to four-fifths saturation. Perchlorate, in amounts such as usually persist after wet digestion, had no effect. Large excess of the salts of the alkali or of the alkaline earth metals, likewise, does not influence the reaction, providing precipitation of hydroxides and phosphates is prevented by the addition of citrate.

Besides that formed with cobalt, well defined coloured N.R.S. co-ordination compounds are formed under similar conditions with nickel, copper, iron and palladium. The properties which were observed are set out in Table 1 and are sufficient to indicate the behaviour of these complexes towards nitric acid and bromine. The effect of citrate on the interaction between these metals and N.R.S. is also indicated. This was used to reduced interference of Fe, Ni and Cu during the estimation of cobalt. Van Klooster's observation (1921) that iron, nickel and copper N.R.S. complexes are decomposed in boiling nitric acid, was confirmed. While acid conditions favour its formation, the brightly coloured Pd–N.R.S. compound tends to

fade on boiling in 1·0N. HNO_3, and the solution is rendered colourless when brominated after boiling in nitric acid solution.

TABLE 1.

	In Acetate at pH 7·0.	In Citrate pH 7·0.	Effect of subsequent boiling 1·0N. HNO_3.	Effect of subsequent bromination.
Nitroso R. Salt	Greenish yellow soln.	Greenish yellow soln.	Yellow soln.	Colourless soln.
Cobalt + N.R.S.	Brilliant red soln.	Brilliant red soln.	Brilliant red soln. (unaffected)	Brilliant red soln. (unaffected)
Nickel + N.R.S.	Greenish brown soln.	Brown soln. (slow reaction)	Yellow soln. (Ni–N.R.S. decomposed)	Colourless soln.
Copper + N.R.S.	Green precipitate	Olive green soln.	Yellow soln. (Cu–N.R.S. decomposed)	Colourless soln.
Iron + N.R.S.	Brown soln.	No immediate reaction	Yellow soln. (Fe–N.R.S. decomposed)	Colourless soln.
Palladium + N.R.S.	Rose red soln.	Rose red soln. (slow reaction)	Rose red soln. Intensifies in acid. Fades on boiling	Colourless soln.

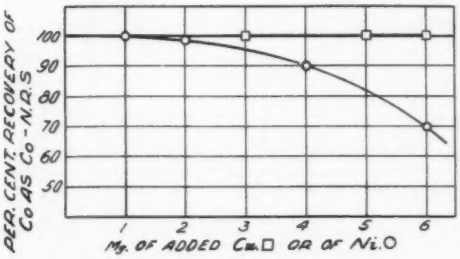

Fig. 3. The influence of Ni. and of Cu. on the formation of Co–N.R.S. from 30 γ cobalt plus 2 mg. N.R.S. in 0·2 M. citrate at pH 8·0.

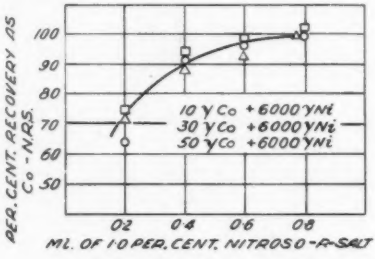

Fig. 4. The influence of increased N.R.S. concentration on the interference by Ni. in the formation of Co–N.R.S. in 0·2 M. citrate at pH 8·0.

To study the interference of other metals in the interaction with cobalt, the amount of N.R.S. in various reacting systems was reduced to about 30 p.c. excess of that required to combine completely with the cobalt present, and the influence of various metals on the velocity of the formation of Co–N.R.S. was investigated over a range extending from pH 5·0 to pH 8·0 in citrate–phosphate buffers. The rate of reaction was uninfluenced by the presence of $Fe^{'''}$, Mn, Zn, Cd. Pb and Sn when present in ten times the concentration of the cobalt. The formation of Co–N.R.S. is unaffected then by these metals even under most unfavourable conditions.

When the amount of reagent is limited, the presence both of copper and nickel influences seriously the rate of the formation of Co–N.R.S. Thus the colour produced at pH 6·1 in five minutes at room temperature by 20 γ of cobalt was reduced to 10 p.c. of the theoretical in the presence of 200 γ of copper; the reaction, however, proceeded to completion in this time at pH 8·5 and it was 95 p.c. complete at pH 8·1, 82 p.c. at pH 7·4 and 55 p.c. at pH 6·8; nickel interfered similarly. In the absence of citrate, both nickel and copper very seriously compete with cobalt. In acetate buffers, for example, the formation of Co–N.R.S. is reduced to negligible amounts when excess of either nickel or copper is present. (Fig. 5) *When equilibrium is established in the reacting system by boiling for two minutes, the interference by nickel and copper may be overcome, providing the amount of N.R.S. is increased, excess of citrate is present, and the solution is adjusted to pH 8·0 (Fig. 5).*

The recovery of 30 γ cobalt as Co–N.R.S. was observed to be complete under these conditions in the presence of 6,000 γ of copper. Nickel, however, prevented the completion of

the formation of Co–N.R.S. if present in excess of 1,000 γ, (Fig. 3), and the theoretical recovery of cobalt was obtained only after further increasing the concentration of the reagent (Fig. 4).

Interference by copper and nickel evidently originates from competition between the various reacting ions for the N.R.S. In this competition, cobalt is favoured by alkaline conditions and the presence of citrate (Fig. 5). Local concentration of the reagent renders more easy the formation of other N.R.S. complexes with competing ions when these are in great excess. The reagent should, therefore, be mixed rapidly and completely when it is introduced into the assay, by blowing a thin stream of relatively dilute reagents from a pipette into the buffered solution containing the cobalt.

While iron in the presence of excess citrate does not compete seriously with cobalt for the N.R.S., the colour imparted to the solution by ferric citrate when iron is present in large excess interferes with the estimation of the Co–N.R.S. complex. Iron can be removed from solutions containing cobalt by extracting as ferric chloride with ether from 5N. HCl, a procedure originally suggested by Rothe (1892, 1893). The partition of $FeCl_3$ into ether was studied, and cobalt under these conditions was found not to be extracted, thus confirming Speller (1901), Kidson, Askew and Dixon (1936), and McNaught (1939).

This procedure was employed only in special circumstances as the dithizone extraction described below allows a clean separation of cobalt from iron.

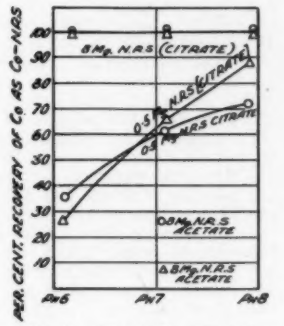

Fig. 5. The influence of reaction and of buffer salts on Co–N.R.S. formation. Equilibria established by boiling 20 γ Co. in the presence of 200 γ Cu Δ or 200 γ NiO.

The separation of cobalt by means of diphenylthiocarbazone. Owing to the minute amount of cobalt usually present in plant and animal tissues, it is desirable to separate the metal from the relatively massive quantity of other elements introduced during the process of wet

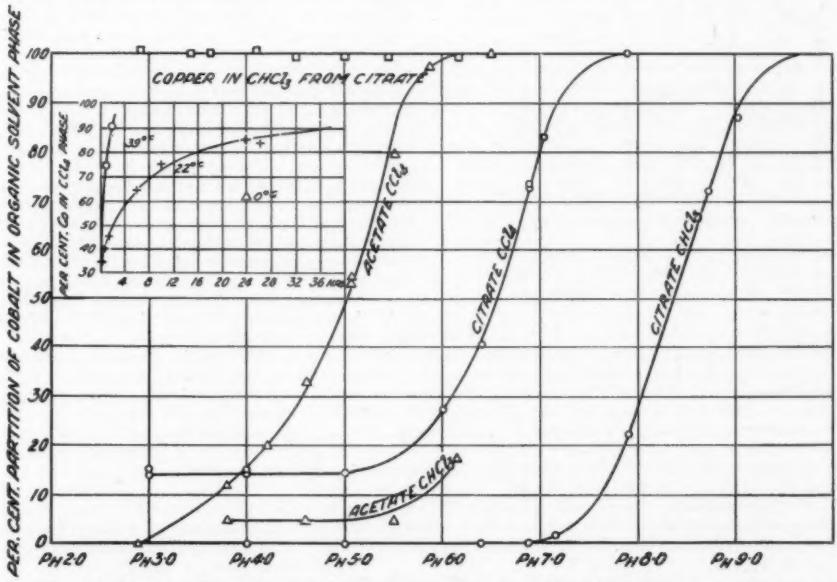

Fig. 6. The influence of reaction of aqueous phase and of buffer salts on partition of cobalt–dithizone complex into organic solvents. 50 γ Co. in 10 ml. 0·02 M. buffer + 10 ml. of dithizone 0·05 p.c. in organic solvent. 24 hours, room temperature.

(Inset) Influence of time and temperature on partition Co–dithizone into CCl_4 from citrate at pH 8·6. Other conditions as above.

digestion. With this purpose in view, some of the properties of the cobalt–diphenylthiocarbazone co-ordination complex were investigated. Since its introduction by Hellmut Fischer, diphenylthiocarbazone (dithizone) has been employed extensively as a reagent for the separation and estimation of micro amounts of many metals. The literature which has been reviewed by Fischer (1937) and by Wichmann (1939) contains, however, very few references to the cobalt complex which are cogent to this problem.

The formation of the cobalt dithizone complex and its partition into either chloroform or carbon tetrachloride was studied over the range pH 2·0 to pH 9·0 with different buffer salts. 10 ml. of the organic solvent containing 5 mg. of dithizone were shaken with 10 ml. of the aqueous buffer solution of 50 γ Co. After standing with occasional shaking, the layers were separated and cobalt estimated in either phase. The recovery rarely deviated more than ± 2·0 p.e. from the total cobalt added to the original aqueous phase.

The final partition is influenced by the reaction, the nature of the organic solvent, and the presence of citrate ions (Fig. 6). It is the resultant of a number of variables, and it would not be expedient to determine exhaustively their separate effects.

To estimate the conditions which may safely be employed in analytical procedures, the influence of some factors was examined further. The partition in the presence of citrate was given especial consideration for, when dealing with tissues, precipitation of the phosphates of calcium, iron, etc., must be prevented during the separation and this is ensured by the addition of citrate prior to neutralization of the acid solution.

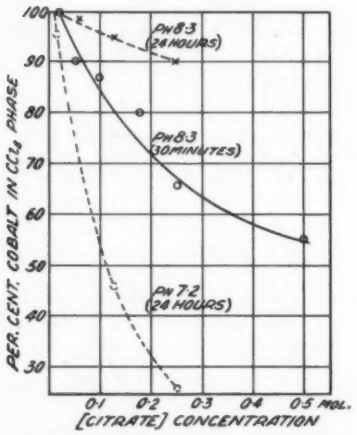

Fig. 7. Influence of citrate concentration on partition of Co–dithizone into CCl_4. Conditions as in Fig. 6.

Partition of the cobalt into the organic solvent phase is depressed by increasing the concentration of citrate (Fig. 7). These data were obtained after standing 24 hours at room temperature with occasional shaking, and thus more or less approximate to true equilibrium. The more rapid establishment of equilibrium may be obtained under working conditions by warming the solution (inset Fig. 6).

Ferric iron does not react with dithizone and is not extracted under these conditions by the organic solvents employed; by the judicious use of citrate and a chloroform solution of dithizone, most of the other metals that react with dithizone may be extracted at pH 5·0 without any removal of cobalt which subsequently may be extracted quantitatively with a carbon tetrachloride solution of dithizone at pH 8·5.

The method: For the destruction of organic matter, oxidation by wet digestion with nitric, perchloric and sulphuric acids is to be recommended as this involves no loss of cobalt through its sublimation as the chloride or through its combination with siliceous residues. The bulk of the residual acids should be removed, as the addition of massive amounts of alkali to adjust the reaction may introduce extraneous cobalt, and a high concentration of neutral salts interferes with the formation and partition of the dithizone–metal complexes.

To ensure that the calcium, magnesium, iron and heavy metals remain dissolved when the solution, which contains phosphate, is rendered alkaline prior to the extraction of cobalt, sufficient citric acid is added to produce a final concentration of 0·03 M. citrate in the aqueous phase. There is no partition of cobalt into chloroform-dithizone solution from this concentration of citrate unless the reaction is distinctly alkaline, whereas the greater bulk of other heavy metals may be extracted as dithizone complexes by chloroform from quite acid solution. A preliminary extraction with a solution of dithizone in chloroform at a reaction between pH 3·0 and pH 4·0 is to be recommended.

When adjusting the reaction of the solution, *it is essential that excessively alkaline conditions be avoided* and a soda solution buffered with phosphate and borate is utilized for this purpose. The titration curve of this solution against citric acid and hydrochloric acid is set out in Fig. 8.

Cobalt in aqueous citrate solution at pH 8·0 combines with dithizone, and at this reaction the complex parts readily into carbon tetrachloride. Ferric iron is not extracted. The cobalt may be recovered quantitatively after the extract has been freed from the carbon tetrachloride and the residue oxidized by wet digestion.

The procedure to be adopted for adjusting the reaction of this final digest prior to the production of the Co–N.R.S. complex calls for some comment. If an internal indicator is to

be utilized for this purpose it should be colourless in the acid solution resulting after the addition of nitric acid. Phenolphthalein meets these requirements, but if it is present in relatively dilute systems containing cobalt and nitroso R. salt, the colour intensity of the resulting Co–N.R.S., after the solution has been boiled with nitric acid and brominated, no longer obeys Beer's Law; nor is it possible to obtain colourless blanks in the absence of cobalt if phenolphthalein be present. Phenolphthalein, o-cresol phthalein and thymol phthalein all

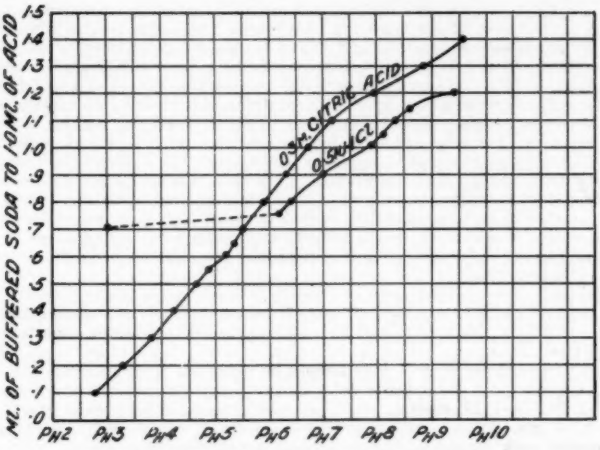

Fig. 8. Titration curves of buffered soda.

form coloured compounds when boiled in nitric acid with N.R.S., *and these extraneous colours persist in quantity sufficient to interfere very seriously with the colorimetric estimation of the Co–N.R.S. complex, even after bromination.* The red compound formed when N.R.S. reacts with phenolphthalein in part accounts for some of the results reported by workers who have employed the procedures specified by Kidson, Askew and Dixon (1936) and by McNaught (1939) both of which call for rather free use of phenolphthalein.[1] Extension of the search for suitable indicators led also to quinoline blue being discarded. For this reason, *the employment of internal indicators in adjusting the reaction of the final solution is rigorously to be avoided.* The analytical procedure is arranged so that appropriate buffers may be used to adjust the final solution to a reaction suitable for the production of the Co–N.R.S. complex, and so it is necessary to remove completely the residual sulphuric acid remaining after wet digestion of the dithizone extract. This may be achieved by fuming off over a gentle heat and by finishing the evaporation in a muffle at 350° C. High temperatures are to be avoided as the anhydrous cobalt sulphate decomposes at 850° C. and may then, in part, combine with the vessel. The residue dissolves readily in dilute citric acid and the reaction then may be adjusted with the calculated amount of the other buffer component. Citrate is again employed at this stage as its presence not only prevents the precipitation of cobalt hydroxide at pH 8·0 but also overcomes completely the possibility of interference by nickel. A considerable excess of nitroso R. salt is used and the residual yellow colour of the reagent is discharged by bromination in nitric acid solution. The cobalt N.R.S. complex may then be estimated by direct colorimetry.

The reagents: While cobalt is not a common contaminant of the reagents employed in the procedure, it is essential to redistil the nitric acid and the sulphuric acid. The other reagents should be of the highest quality and carefully tested for cobalt. All water should be redistilled in hard glass apparatus. One filtration only is called for and for this, filter paper that has been soaked in 10 p.c. HCl, washed thoroughly and dried, should be employed.

The following reagents are required:

Nitric acid (S.G. 1·42): Reagent quality redistilled from all glass apparatus.

Sulphuric acid: Reagent quality redistilled from a Pyrex retort.

Perchloric acid (S.G. 1·54): Highest grade analytical reagent.

[1] Since this was written, McNaught & Paul (N.Z. Jour. Sci. & Tech., 1939, *21*, 95) have recognized the error introduced by the use of phenolphthalein in the methods of McNaught (1939) and of Kidson, Askew and Dixon (1936).

0·2 M. Citric acid: Prepared from the highest grade reagent and standardized by titration with carbonate-free NaOH (Phenolphthalein).

Normal soda: Prepared from highest grade reagent NaOH freed from carbonates, dissolved in redistilled water.

Buffered soda: Boric acid 6·184 gm. (0·1 mol.), $Na_2HPO_4.2H_2O$, Sorenson's salt 35·62 gm. (0·2 mol.), standardized 1·0N sodium hydroxide 500 ml., redistilled water to 1 litre. (The titration characteristics of this solution are set out in Fig. 8.)

Dithizone solutions: Dithizone 0·2 p.c. in chloroform and dithizone 0·05 p.c. in carbon tetrachloride. These solutions should be prepared with redistilled solvents. The chloroform is best purified by distillation after the addition of 1 p.c. of alcohol. The dithizone should be freed from oxidation products and heavy metals by the procedure of Fischer (1937). The dithizone in CCl_4 may be prepared as follows: Dissolve 0·5 gm. of dithizone in 500 ml. of redistilled CCl_4. Transfer to a 1-litre separating funnel and add 250 ml. of 1 p.c. by vol. of redistilled ammonia S.G. ·880 in water and shake. The free dithizone passes into the aqueous phase. Wash twice with about 100 ml. of carbon tetrachloride; the aqueous phase is then rendered acid by the addition of 3·0 ml. of redistilled sulphuric acid and the dithizone is shaken out into 600 ml. of CCl_4. The CCl_4 phase is separated and washed twice with redistilled water. It is then diluted to 1 litre and transferred to a dark glass bottle. The chloroform solution is prepared similarly. Both solutions are relatively unstable, but deterioration is not great if they are stored in a dark ice box. The permanency of dithizone in chloroform is increased by the addition of 1 vol. p.c. of alcohol, and in carbon tetrachloride by overlaying the solution with dilute aqueous sodium sulphite (Clifford, 1938).

Reaction indicators: Brom phenol blue (40 mg. in 100 ml. water containing 5·7 ml. of 0·01N. NaOH); methyl red (25 mg. in 100 ml. of 60 p.c. alcohol and phenolphthalein (50 mg. in 100 ml. of 50 p.c. alcohol), cresol red (40 mg. in 100 ml. water containing 10·5 ml. of 0·01N. NaOH).

Nitroso R. salt: The nitroso R. salt may simply be prepared by dissolving 0·1 mol. (35 gm.) of pure 1 naphthol 3·6 disulphonic acid, R. salt, in about 400 ml. of water acidified with 10 ml. of HCl S.G. 1·19, cooling to 10° C. and adding drop by drop over a half an hour, 0·1 mol. (7·0 gm.) of sodium nitrite dissolved in 25 ml. of water, keeping the reaction mixture cooled with ice. The yellow crystals are filtered and washed with ice water and finally with cold alcohol. The yield which depends on the extent of washing usually exceeds 70 p.c. of theoretical. A 0·2 p.c. solution in water is used. This is stable.

Bromine solution: Water saturated with bromine is separated from excess. The concentration of bromine is estimated by titration with thiosulphate after the addition of excess of KI. It is then diluted to 0·2N. This solution is not stable.

Standard cobalt solution: A solution containing 1 γ Co per ml. should be freshly prepared by diluting a stock solution of cobalt nitrate containing 1 mg. Co per ml.

The Procedure for Analysis:

Preparation of material: The plant or animal tissue is dried at 100° C. Approximately 10 gm.,[2] accurately weighed, is transferred to a 500 ml. Kjeldahl flask, 80 ml. of nitric acid (S.G. 1·42), 5 ml. of perchloric acid (S.G. 1·54) and 3 ml. of sulphuric acid are added and the mixture warmed gently until the onset of a vigorous reaction is indicated by the evolution of dense nitrous fumes. The flask is then removed from the heat source and stood overnight. Heating then is continued until the contents of the flask are concentrated to about 3 ml., more nitric acid being added if necessary to ensure the complete oxidation of the organic matter. The contents are diluted with approximately 10 ml. of water, boiled for a few minutes, filtered through a prepared paper and washed into a silica basin. The water is cautiously evaporated and all but a faint trace of sulphuric acid removed by fuming off over a steady heat. It is essential to leave a trace of sulphuric acid at this stage in order to prevent the formation of insoluble iron oxides from the ferric sulphate which decomposes at 480° C. Precipitates at any stage of the analysis are to be avoided owing to the tendency of cobalt to be adsorbed. The residue is taken up in 7·5 ml. of 0·2 M. citric acid and diluted to about 30 ml.

Extraction with dithizone: Five drops of brom phenol blue are added to the resulting solution and 1·0 N. NaOH is run in, drop by drop, until a distinct greenish-blue colour appears through the yellowish tint imparted by the ferric citrate. *The solution should still be acid to methyl red.* It is then washed completely into a 100 ml. separating funnel and diluted to 50 ml. in order to adjust the citrate concentration to 0·03 M. This solution is extracted with 0·2 p.c. dithizone in chloroform, 20 ml. portions being added, the contents vigorously shaken and the chloroform layer separated off. The first extracts usually are a cherry red colour. The operation is repeated until the chloroform layer retains the original green colour of the dithizone solution. Whereas three such extractions usually suffice, it is desirable to continue until the dithizone is no longer exhausted by combination with heavy metals in the aqueous

[2] Or sufficient to provide between 1 γ and 5 γ Co. If less is used, reduce accordingly the quantity of reagents for oxidation.

phase. The solution is then washed once with redistilled chloroform. The copper is extracted quantitatively and may be estimated in the combined chloroform extracts by the procedure outlined below. The reaction of the aqueous phase is adjusted to about pH 8·3 by adding a few drops of phenolphthalein while still in the separating funnel and cautiously titrating with buffered soda until the first sign of purplish pink colour appears. For this about 6·0 ml. of the buffered soda will be required. A good light and a white background are necessary to detect these colour changes. The cobalt is then removed by successive extractions with 10 ml. portions of 0·05 p.c. dithizone in carbon tetrachloride. Three extractions usually are sufficient but again the extractions should be continued until the carbon tetrachloride phase retains the green colour of the original dithizone solution.

Treatment of the dithizone extract: The combined carbon tetrachloride extracts contain the whole of the cobalt. The solvent is distilled off from a Pyrex boiling tube (about 2·5 cm. × 16 cm.) and the residue ignited with 1 ml. nitric acid, 0·2 ml. sulphuric acid and 0·5 ml. of perchloric acid, until quite colourless. The contents of the tube then are washed into a silica basin, and after evaporating the water, the *sulphuric acid is fumed off completely.* The basin should, if possible, be heated for five minutes in a muffle at 350° C. to ensure the total removal of the free sulphuric acid. Excessive temperatures are to be avoided.

The production of Co–N.R.S. and its colorimetric comparison: The residue is dissolved in 1 ml. of 0·2 M. citric acid, washed back into the boiling tube with a minimum of water. If the total volume is in excess of 5 ml., it should be reduced by evaporation of the acid solution. The reaction is adjusted cautiously by adding exactly 1·2 ml. of buffered soda (cf. Fig. 10). A small drop may be withdrawn and the reaction checked with cresol red. The Co–N.R.S. complex is developed by introducing 1 ml. of 0·2 p.c. N.R.S. with a fine pipette while shaking vigorously. The solution is then boiled for 1 minute. The Co–N.R.S. is stabilized by adding 1·0 ml. of concentrated HNO_3 (S.G. 1·42) and again boiling for a minute. The yellow colour of the excess reagent is discharged by adding 0·5 ml. of 0·2 N. bromine to the warm solution and allowing to stand 5 minutes, the excess bromine subsequently being removed by boiling for a minute. Even ebullition is assured if a glass boiling stick is employed.

The solution is cooled, diluted to 10 ml. and the intensity of the red colour compared against a standard prepared by adding the required amount of cobalt to 1 ml. of 0·2 M. citric acid, adjusting the reaction with 1·2 ml. of buffered soda, developing the Co–N.R.S. stabilizing with nitric acid and discharging the excess reagent by the above procedure and diluting to 10 ml.

The estimation of copper: The residue from the chloroform-dithizone extracts after evaporation of the organic solvent is digested with 2 ml. of sulphuric acid and 0·5 ml. of perchloric acid until colourless. The acid digest is then washed out and diluted to 50 ml. with glass distilled water. An aliquot containing between 5 γ and 15 γ of copper is transferred to 100 ml. stoppered cylinder, rendered alkaline with redistilled ammonia and made up to 50 ml. Sodium diethyl dithiocarbamate (0·5 ml. of 1 p.c. aqueous solution) is added, the yellow copper complex extracted by shaking with 5–10 ml., accurately measured, of pure amyl alcohol and comparison made with appropriate standards. This procedure is based on the method of Sylvester and Lampitt (1935).

SUMMARY.

The red association complex formed by the interaction of cobalt with naphthoquinone (1, 2)-oxime (1) disulphonic acid (3·6), (nitroso R. salt) is unaffected by bromine in relatively concentrated nitric acid, whereas the colour of the free N.R.S. is entirely discharged by bromination in nitric acid solution.

In systems containing cobalt and an excess of over three molecular equivalents of N.R.S., the cobalt complex is formed rapidly and completely at reactions between pH 5·5 and pH 8·5. The velocity of formation is less rapid at reactions more acid than pH 5·5, and at pH 2·5 cobalt and N.R.S. do not combine.

If the red Co-N.R.S. complex is stabilized in N. nitric acid and the yellow colour of excess N.R.S. is discharged by bromination, the molecular extinction coefficient of the red complex is constant over a wide range of cobalt concentrations.

The intensity of the colour of the Co-N.R.S. complex is such as to allow precise colorimetric comparison to be made between solutions containing the complex formed from 1 γ Co in 10 ml.

Copper and nickel, when in concentrations over 100 times that of cobalt tend, by similar complex formation, to compete with cobalt for the N.R.S. The effect may be overcome completely by the addition of citrate and by increasing the relative amount of N.R.S. and carrying out the reaction at pH 8·0. Other elements

in concentrations in considerable excess of those likely to be encountered in biological materials do not interfere with the complete recovery of cobalt as Co-N.R.S.

The coloured ferric ion, when present in amounts that normally occur in plant and animal tissues, seriously affects colorimetric estimation of the Co-N.R.S. The separation of iron from cobalt by extraction of $FeCl_3$ with ether from 5N. HCl is discussed.

While cobalt in 0·03 M. citrate solution, when shaken with dithizone in chloroform, does not part into the organic solvent until the solution is strongly alkaline, copper and the bulk of other dithizone metals may be separated by this means at pH 4·0. Cobalt may subsequently be extracted quantitatively at pH 8·0 by extraction with dithizone in carbon tetrachloride, and may be recovered by igniting the residue from the combined carbon tetrachloride extracts.

A method for the estimation of the minute amounts of cobalt present in plant and animal tissues has been based on the above facts, and the procedure demonstrated to be capable of estimating one microgram of cobalt in 10 gm. of dried tissue with an accuracy exceeding $\pm$ 5 p.c. The recovery of 2 γ of cobalt is quantitative within the $\pm$ 1 p.c. error inherent in direct colorimetry.

REFERENCES.

Clifford (1938): J. Assoc. Offic. Agr. Chem., 21, p. 695.
Fischer (1937): Angew. Chem., 50, p. 919.
Hoffmann (1885): Ber. 18, p. 46.
Ilinski and Knorre (1885): Ber. 18, p. 699.
Kidson, Askew and Dixon (1936): N.Z. J. Sci. & Tech., 18, p. 601.
v. Klooster (1921): J. Amer. chem. Soc., 43, p. 746.
Marston (1939): Ann. Rev. Biochemistry (California), 8, p. 570.
McNaught (1939): Analyst, 64, p. 23.
Rothe (1892): Stahl u. Eisen, 12, p. 1052.
Rothe (1893): *Ibid.*, 13, p. 333.
Speller (1901): Chem. News, 83, p. 124.
Stare and Elvehjem (1932): J. biol. Chem., 99, p. 473.
Stenhouse and Groves (1877): J. chem. Soc., Pt. 2, p. 47.
Sylvester and Lampitt (1935): Analyst, 60, p. 376.
Wichmann (1939): Ind. and Eng. Chem. (Anal.), 31, p. 66.

INFLUENZA VIRUS INFECTIONS OF THE CHICK EMBRYO BY THE AMNIOTIC ROUTE

1. GENERAL CHARACTER OF THE INFECTIONS.

by F. M. BURNET[1]

(From the Walter and Eliza Hall Institute, Melbourne).

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In a preliminary paper (Burnet, 1940) an account has been given of a method of inoculation by which easily recognizable influenzal infections can be produced in the lung of the chick embryo. A more detailed account of the method and of the nature of the lesions produced is now presented.

Soon after the influenza virus was isolated, it became evident that the virus exerted primary pathogenic action only on the epithelium of the respiratory tract. Francis and Stuart-Harris (1938) found that when ferrets were infected intranasally the respiratory epithelium of the nasal cavities was very severely damaged, but that adjacent olfactory epithelium was, except rarely, wholly unaffected. No evidence has been produced that the virus can multiply in a free-living animal anywhere but in the epithelium of the respiratory tract. Multiplication occurs on the chorioallantois of the developing chick, and most strains can more or less easily be adapted to produce well-defined lesions whose primary location is the ectodermal epithelium of the chorion. We could not obtain infection of the chorioallantois with filtrates of throat washings from influenza patients, although Francis and Magill (1937) record positive results. As far as we are aware, no workers have obtained recognizable lesions on the egg-membrane except after a considerable number of passages. This considerably limits the usefulness of chorioallantoic methods in influenza research.

Although retrospectively it seems an obvious possibility that unadapted influenza virus might produce lesions in the lung of the developing chick, the method was evolved rather accidentally in the course of other experiments. These were concerned in determining the way by which generalized infections of the embryo resulted from chorioallantoic infections. It was found that with unadapted mouse-virus strains invasion of the embryo occurred in very young embryos, but not in those nine days or older at the time of inoculation. No evidence (by titrations in mice) could be obtained of any specific localization in the developing lung. With a fully adapted egg-strain, "Melbourne egg", which is lethal for the embryo in two days, rapid invasion within twenty-four hours occurred in eggs inoculated from the seventh to the fifteenth day of incubation. In none was there any evidence of specific localization in the lungs. While these experiments were in progress, Buddingh and Polk's paper (1939) on meningococcal infections of the chick embryo suggested the practicability of amniotic inoculation. With this method it was found that after infection with mouse-pathogenic strains, unadapted or partially adapted to chorioallantoic propagation, a much higher content of virus was found in the lung than in any other organ. Most of the available influenza virus strains were then tested and histological examinations made of the embryo lungs. The presence of large numbers of desquamated cells in the various subdivisions of the bronchial tree suggested the use of smears from the tracheal fluid as an index of infection, a method which has proved extremely useful.

We have been unable to test material from human infections directly, but we had available glycerolated material from ferrets used for the primary isolation of strains in the epidemic of July, 1939. The turbinates had been removed about eight months previously and stored in a mixture of equal parts of glycerol and Ringer's solution at about — 4° C. All four strains of virus isolated in the Melbourne 1939 epidemic, "Bundoora", "R", "H", and "W" (Burnet and Lush, 1940), have been tested as first-passage ferret material and shown to be capable of producing typical lesions. The strains have been maintained by amniotic passage for varying numbers of generations. Proof that the agent transmitted from egg to egg was actually influenza virus was obtained by showing that typical ferret infections could be obtained after many passages, and that the serum of the ferrets thereafter neutralized the "Melbourne" virus both on the chorioallantois and in the mouse.

[1] Work carried out under grants for research on virus diseases from the National Health and Medical Research Council and from Mr. E. Alec Cato.

Technique. The procedure of amniotic inoculation has been already described (Burnet, 1940) and a brief account only will be given here. For all routine work White Leghorn eggs incubated for twelve days are used. The position of the embryo is determined by "candling" and an oval area about 2 cm. by 1·5 cm. marked for drilling. The chorioallantoic membrane is dropped by the usual technique (Burnet, 1936) and the shell membrane clipped away over the oval area. A slit is made with scissors in the chorioallantois over the embryo and the amnion drawn upwards with fine-pointed forceps. The inoculation is made into the amniotic cavity with a capillary pipette drawn out to a sharp point; the preliminary injection of a bubble of air shows that the cavity has been entered. The opening is sealed with a coverslip resting on a rim of vaseline-paraffin mixture and the egg returned to an incubator at 35° C. for two to four days.

Infection with virus by this method results in death of the embryo after a period varying with the amount administered and the virulence of the strain. Owing to the speed with which *post-mortem* autolysis occurs in the embryo it is advisable to open the eggs for examination about a day before death is expected. As a routine some of the allantoic and amniotic fluids are collected aseptically and cultured for sterility; the embryo is then removed to a sterile Petri dish. The larynx and trachea are exposed by cutting through the mandibular articulation on both sides, and the tracheal fluid removed by aspiration with a fine-pointed capillary pipette. Infected embryos show a turbid fluid. Smears are best made by transferring the fluid to a capillary, sealing one end and centrifuging. The deposit is made into a thin smear and stained by Leishman's method. The lungs are then removed and examined macroscopically, and if necessary fixed in neutral formol-saline for histological section.

When organs are titrated for their virus content, the routine has been to grind the two lungs with 1 c.c. of broth, centrifuge and use the supernatant fluid for test undiluted or diluted serially in saline containing 10 p.c. normal horse-serum. For other organs, usually brain and liver, a portion roughly equal in size to the two lungs was similarly ground with 1 c.c. of broth. To avoid contamination with amniotic fluid the embryos were washed with 50–100 c.c. of saline before opening the abdominal cavity, and the individual organs washed twice in 10 c.c. of broth or saline before being ground with quartz powder.

Symptoms and macroscopic appearances. The following description is based mainly on the behaviour of unadapted ferret-strains, but is equally applicable to the mouse-strains "Melbourne", "W.S." and "Bur.", and to sub-strains of "Bur." and "R" partially adapted to the chorioallantois but not yet lethal for the embryo. The behaviour of fully-adapted egg-strains "Melbourne egg" and "W.S. egg" differs considerably, and will be described subsequently.

After a fairly large dose of virus, it is rare to find an embryo normally vigorous when the egg is opened three days later. A diminished blood flow in the membranes is shown by the smaller calibre of the vessels and a reduction in the warm background tint due to the capillary bed. There is a lack of active movement, an anaemic and often slightly jaundiced appearance of the embryo, and often a shrunken dehydrated appearance. Almost always the amniotic fluid is considerably reduced in amount, and the embryo may be tightly compressed by the amnion, the cavity being quite empty of fluid. It is particularly in such cases that the embryo appears shrunken and dehydrated. Since, as will be shown later, the specific structure of the lung is completely disorganized, it is obvious that the chick must die before hatching, but the actual time of death varies greatly with circumstances. With the ferret-strains "Bundoora" and "R" in their first or second amniotic passages death may be delayed for at least six days, and with small doses of virus the embryo may show almost normal development with small pale lungs as the only evidence of infection. After a few passages early deaths become much more frequent, even with high dilutions. Thus the twenty-third amniotic passage of "Bundoora" was titrated using pooled amniotic and allantoic fluids as stock virus and inoculating three eggs each with dilutions from 10^{-3} to 10^{-7}. At sixty-eight hours after inoculation only two embryos were alive, one with 10^{-6} and one with 10^{-4}; both showed typical signs of infection. Since the others had been alive twenty-four hours previously and were free of bacteria on culture, the deaths were clearly a specific result of infection. Many fluids from dead embryos have been tested for their virus content; in no instance have we failed to find virus in an embryo dying more than forty-eight hours after inoculation and free from bacterial infection. With strong virus specific deaths may occur within forty-eight hours, but owing to the occasional occurrence of traumatic deaths we have disregarded these early deaths unless subinoculation proved them specific. We have not observed any specific *post-mortem* appearances in these dead embryos; the haemorrhagic lesions in brain or muscles characteristic of a lethal strain fully adapted to the chorioallantois have never been seen. There is general congestion of the viscera, and if death is very recent the characteristic lung changes may be recognized. *Post-mortem* autolysis takes place very rapidly, however, and as a rule definite proof that an embryo has died of specific infection can only be obtained by titrating the virus content of the fluids.

As a routine the eggs are examined first at about 44 hours after inoculation, and the proportion of deaths noted by observation through the cover-glass seal. Dead embryos and those expected to die within 24 hours are then examined. Eggs receiving higher dilutions are opened later, the aim being to allow as much time as possible for lesions to develop without killing the embryo. The most important sign of infection is the presence of turbid tracheal fluid, and as a general rule any macroscopically visible turbidity not due solely to accidental bleeding is pathognomonic of specific infection.

The normal appearance of the lung changes considerably during the period over which the eggs are opened, i.e. from the thirteenth to the seventeenth days of incubation. In the early stages it is pale and easily separated from the thoracic wall. At about the fifteenth day the colour deepens rapidly to a fairly dark red, the attachment to the chest-wall becomes stronger and the texture changes to a tougher and flabbier consistence. These normal changes and the fact that embryos of the same nominal age may not have developed to the same degree must be kept in mind in assessing the appearances in infected embryos. In embryos examined two or three days after infection, i.e. at fourteen or fifteen days incubation-age, the lungs appear paler, somewhat larger, and often strikingly firmer than normal. With fully developed lesions the colour may be definitely creamy, and occasionally there are regions covered with small punctate haemorrhages. If the still surviving embryo is examined four, five or six days after infection, the usual finding is a small pale lung, its colour varying from yellowish-white through nondescript creamy shades to a pale brown, and its texture being moderately tough and flabby.

In practice it is found that a diagnosis of specific infection can almost always be made from the macroscopic appearance of the tracheal fluid and of the lungs. Microscopic examination of the tracheal fluid provides a further check, and only in very rare instances is it necessary to resort to histological section of the lung or subinoculations to establish the diagnosis.

The distribution of virus in the fluids and organs of infected embryos. Titrations of virus content may be made by amniotic inoculation, using serial tenfold dilutions with the proviso that embryos receiving limiting dilutions of unadapted viruses should be kept at least four days before being examined. If strains producing well-developed lesions in the mouse-lung or on the chorioallantois are being studied it may be more convenient to use these alternative methods of titration.

In an attempt to follow the course of infection it is first necessary to know the immediate distribution of the inoculum placed in the amniotic cavity. Trypan blue (1 p.c.) was administered in the usual way to three embryos and the eggs opened at intervals. At one-and-a-half hours the whole of the amniotic fluid was uniformly coloured; the mouth and at least the upper part of the trachea contained blue fluid. On opening the body cavity the stomach was seen to contain a considerable amount of the dye, but there was none in the intestine. At twenty hours much of the dye in the amniotic fluid was segregated in a mucinous mass. The whole length of the alimentary canal was stained, as was the trachea. The lungs were uncoloured, and the allantoic fluid was also clear of the dye. At forty-four hours there was no appreciable difference.

These results indicate that anything placed in the amniotic cavity is soon swallowed, and thus has an opportunity to enter the trachea. In all probability the movements of the embryo also result in a certain amount of to-and-fro movement of the fluid in the trachea, which would bring virus in the inoculum down to the lung more rapidly than mere diffusion and infection by continuity. As far as we can gather from the literature and from our own experiments, fluid is continually secreted into the amniotic cavity. It is swallowed and passes through the alimentary canal, receiving intestinal and renal secretions *en route*, to pass out into the allantoic cavity through the stalk of the allantois. Here fluid is absorbed, with the result that the allantoic fluid becomes progressively richer in urates and other products of excretion.

The most interesting feature of the titrations of the virus content of fluids and organs is the rapid appearance of large amounts of virus in the allantoic fluid. Table 1 shows the results of three experiments using strain "R" after about twenty passages by the amniotic route, when it had become capable of producing recognizable foci on the chorioallantois. Previous experiments with this strain had shown only negligible amounts of virus in the brain and liver. Table 2 gives similar data for an embryo inoculated with mouse-lung emulsion of strain "W.S." In this case titrations were made by the intranasal route in mice. In both sets of titrations the very high infective titre of the embryo fluids will be noted. Usually

the amniotic fluid has a significantly higher titre than the allantoic, but the opposite is occasionally found, as in the embryo infected with "W.S." (Table 2).

TABLE 1.

Distribution of Virus in Embryos Inoculated Amniotically with Strain "R".

Inoculum and time of examination.	Amniotic fluid.	Tracheal fluid.	Allantoic fluid.	Lung Emulsion.
R. undil. 22 hours	8×10^8	2×10^8	10^8	8×10^4
R. 1: 10^4 22 hours	2×10^7		10^6	2×10^4
„ 46 hours	6×10^7		6×10^7	2×10^6

Titrations of virus by the chorioallantoic method expressed as infective units per c.c.

TABLE 2.

Distribution of Virus in an Embryo Inoculated Two Days Previously with "W.S." (Mouse-Strain).

Material.	Undiluted.	1: 10.	1: 10^2.	1: 10^3.	1: 10^4.	1: 10^5.
Amniotic fluid			44, 45, 46^+		45, 2, 1	2, 2, 0
Allantoic fluid		43, 45, 46^+		44, 47, 45^+	44, 44, 45	
Lung emulsion	45^+, 46^+, 3		3, 2, 2			
Liver emulsion	3, 3, 3					
Brain emulsion	45^+, 46^+, 2					

Mouse-lung lesions are expressed in accordance with the usual convention.

Although we have not finally excluded the possibility that multiplication takes place in the cells lining the amnion, the most likely interpretation of these findings is that virus multiplies in the epithelium of the respiratory tract, especially in the parabronchi of the developing lung. There is a steady discharge of fluid and infected cells from the trachea, and perhaps from the upper respiratory passages. Much of this reaches the amniotic fluid, while part is swallowed and passes continually into the allantoic fluid. When the chick is examined two or three days after inoculation there is no sign of the puncture made in the amnion during inoculation, and we can probably exclude infection of the allantoic fluid across the fused amnion-allantoic membrane.

Of the organs tested the lung has consistently shown larger amounts than any other organ, often many times as much. In Table 3 are collected a series of results obtained by titration of embryo organ emulsions in mice, the three strains used all being adequately pathogenic for titration by this route. The figures shown represent the dilution of stock organ suspension (in 1 c.c. broth) which gave, or by interpolation would give, average 2 + lesions in the mice inoculated. The figures are only approximately accurate, as in most experiments only two mice were used for each dilution, and in several instances the end point was obtained only with a second titration of the same material made after it had been five days in the refrigerator. However, the table shows clearly the excess of virus in the lung and the rather wide variations in virus content in different embryos inoculated with the same material at the same time.

TABLE 3.

Distribution of Virus in Organs of Embryos Infected by the Amniotic Route.

Strain.	Period of infection.	Lung.	Liver.	Brain.
"R" (egg)	2 days	100	10	0·5
	„	10,000	20	1
	„	3,000	200	100
	„	100,000	10	10
	„	2,000	10	10
	3 days	100	10	0·5
"Melbourne" (mouse)	3 days	50	0·5	0
	„	200	0·5	0
"Bur." (mouse)	3 days	2,000	2	1
	„	50	0·2	0
	„	2	0	0
	„	5,000	10	0

Figures represent the dilution giving or calculated to give average 2+ lesions in mice inoculated intranasally.

A few tests have been made with other organs. As would be expected from the movement of fluid, the stomach contains a good deal of virus, and the posterior abdominal viscera show about as much as the liver.

The tracheal fluid as an index of infection. In normal chick embryos the fluid aspirated from the trachea is clear, containing at most only a small flake or two of epithelium. The amount obtainable increases rapidly with growth from the thirteenth to seventeenth days. On centrifuging, no deposit is obtained as a rule. When any deposit is present smears show only red cells with sometimes small masses of epithelium and debris. There are no isolated cells apart from erythrocytes, and none of the specific changes in the epithelium which are characteristic of influenza virus infection.

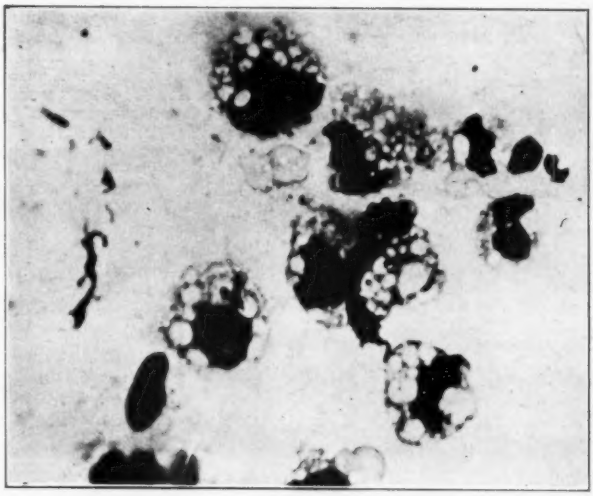

Fig. 1. Typical appearance of cells in smear from tracheal fluid of infected embryo two days after amniotic inoculation.

Stained Leishman x 970.

In embryos with typical infections, forty-eight hours after inoculation the amount of fluid obtainable is often less than normal; it is always visibly turbid, although the opacity may vary within wide limits. Under a lens the appearance is always more or less granular. As a routine, smears from the centrifuged deposit have been stained by Leishman's method. In a typical smear there are several types of cell dispersed singly or in small clumps. Most characteristic are the large vacuolated epithelial cells, several of which are shown in Fig. 1.

These cells appear from 12–20μ in diameter in a thin smear, with abundant protoplasm usually staining light blue and an oval nucleus often distorted by vacuolation. The vacuoles involve both cytoplasm and nucleus and are of very varied size and distribution. Occasionally one sees a very large vacuole with the nucleus and a rim of cytoplasm forming a signet ring appearance, but the multiple vacuoles such as are shown in Fig. 1 are much more commonly seen. The vacuoles often appear to be empty, but may show a variety of apparent contents. The most usual is a pink-staining, finely granular material, within which more definite and deeper-staining granules can be seen. These large cells are phagocytic and may contain debris from other cells, the nature of which can sometimes be recognized, e.g. a red cell nuc-

leus. Their phagocytic character renders it difficult to be certain whether the "inclusions" within the vacuoles are of intrinsic or extrinsic origin. Well-stained specimens are strongly reminiscent of the cytoplasmic appearances in monocytes from the subcutaneous fluid of a canary infected with canary-pox (Burnet, 1933), and the simplest explanation of the appearances would be to consider the vacuoles as surrounding micro-colonies of influenza virus, the pink-staining material being partially-aggregated virus. More refined histological methods and comparison with lesions produced by other viruses and non-living agents would be necessary before this interpretation could be fully substantiated. The only comparative material we have available at present is in smears from infections with the virus of laryngotracheitis of fowls. These show a proportion of cells with highly vacuolated protoplasm, but their appearance differs considerably from that of typical influenza virus cells.

A characteristic feature of these tracheal smears is the presence of primitive cells with fragmented nuclei and often with one or two cytoplasmic vacuoles. They can also be clearly seen in many sections of infected lungs, and are evidently derived from the parabronchi. These cells show what is perhaps a misleading resemblance to primitive red cells, undoubted examples of which are also present in most smears. The general impression received is that the cells which show evidence of specific damage, nuclear fragmentation and vacuolation of cytoplasm and nucleus, can be arranged in two series which seem to converge in a primitive cell with a relatively small amount of deep blue cytoplasm. On the one hand we have the red cell series, and on the other a series becoming progressively larger and more lightly stained till we reach the typical "vacuolated epithelial cell" described above. We have insufficient experience of embryonic pathology to venture any interpretation of this apparent convergence.

The only other cell-type to require mention are the granular leucocytes. These are relatively rare at two days, but when the infection is prolonged, or when older embryos are infected, they become the dominant feature in smears. The appearance varies according to the age of the embryo, the granules becoming more eosinophilic and more like the well-defined rods of the adult with increasing age of the embryo. In infected embryos of total age fifteen to sixteen days, they show a bilobed or kidney-shaped nucleus and a vaguely granular or mottled cytoplasm of a brownish tint. Early forms are occasionally seen, and these seem more prone than the typical leucocytes to show vacuolation.

With a large inoculum the tracheal fluid may contain typical vacuolated cells at twenty-four hours, and will always show them at two days. Particularly with unadapted strains, very small inocula may produce positive findings only after four, five or six days in the form of a few vacuolated cells and leucocytes. In a titration to determine the end-point of infection by the amniotic route it is therefore necessary to use some judgment as to when the embryo should be examined. In an embryo more than a few hours dead the vacuolated cells appear to disintegrate rapidly and smears are obscured by desquamated tracheal cells. It is fairly safe to assume that a death delayed beyond forty-eight hours is specific, but such an assumption will certainly be occasionally in error, and it is always desirable to check the diagnosis with a satisfactory smear. The ideal is clearly to examine the embryo just before it dies, and in practice we generally open all the remaining eggs of a dilution in which a specific death has occurred.

Histological appearances in the lung. The histological changes are naturally complicated by the fact that they occur in a rapidly developing organ, the fine structure of which changes greatly during the period of development in which we are interested—twelve to seventeen days.

At the beginning of this period the parabronchi are in the form of simple tubes of epithelium: about the thirteenth or fourteenth day saccules bud out from these tubes into the interstitial tissue and grow rapidly to form the pulmonary canalieuli by fusion with others from adjacent parabronchi about the seventeenth day. In these five days therefore there is a striking change in the normal appearance from a series of simple tubular structures embedded in a large amount of loose mesenchymal tissue to a densely cellular structure of respiratory epithelium with a minimum of stroma arranged more or less hexagonally at the margin of the areas corresponding to each parabronchus.

When embryos are inoculated at the standard age of twelve days' incubation, the first visible sign of infection is the appearance of areas of irregular epithelial proliferation in the walls of the parabronchi or in the early saccular outgrowths from these. Nuclear changes, in particular fragmentation of the nucleus, are always visible in some cells, sometimes in a large proportion. These proliferative changes extend to all parabronchi, and desquamation into the lumen commences. At this stage larger vacuolated cells are visible, and often contain phagocytized fragments of cells with disintegrated nuclei. Eventually the integrity of the epithelium is completely destroyed and the site of each parabronchus marked by a loose-packed

collection of degenerate cells and leucocytes. The interstitial tissue contains many more cells than normal of varied types: leucocytes, large mononuclear cells, some of which appear to be derived from the saccular epithelium, and fibroblasts. Rarely there are haemorrhages into the interstitial tissue, but it is usual to see some evidence of irregular proliferation of capillary endothelium. In the final stage just before death occurs there is complete destruction of all the epithelial structures except for the lining of the larger bronchi. This shows only minor damage.

When the embryo survives four or five days after inoculation with a small concentration of virus, the pale atrophic lungs show very varied histological appearances. In all there is great disorganization with loss of most of the respiratory epithelium. In some the remains of the parabronchi appear as relatively large open spaces without outgrowths and lined only by a layer of thin endothelial-type cells. The rings of smooth muscle around the parabronchi can often be clearly seen. Other specimens show a medley of partially disorganized structures embedded in an interstitial tissue infiltrated with leucocytes. There is nearly always some evidence of healing in these older lesions, and often very little indication of active virus multiplication. The lining of the larger bronchi usually appears normal.

The lesions in the embryo lung thus summarily described obviously provide a particularly interesting example of embryonic pathology on which much more work could profitably be done. We hope to continue the investigation of the histological conditions in collaboration with Dr. E. Weston Hurst.

DISCUSSION.

The chief aim of this paper is to present evidence for the claim that unadapted influenza virus strains can infect the respiratory tract of the chick embryo and produce easily recognizable effects. The virus strains for which this has been established include the following:

A. First ferret passage strains: "Bundoora", "R", "H", and "W".
B. Stock mouse passage strains: "W.S.", "Melbourne", "Bur.", "R", and "Swine".
C. Chorioallantoic passage strains: "W.S.", "Swine", "Bur.", and "R".

The only virus which has failed to produce the specific lesions described above is the fully-adapted chorioallantoic strain "Melbourne egg".

It appears legitimate to expect that influenza virus could readily be isolated from human throat washings by this technique, and that the method could therefore very largely replace the ferret in influenza virus research.[2] If this is correct, a large field for useful work on the human disease is opened up on such matters as the quantitative changes in the amount of virus liberated at different periods of the disease, the existence of carriers, and the presence of influenza virus in respiratory infections during non-epidemic periods.

After six months' experience, covering several thousand amniotic inoculations, we can be definite that the technique is no more difficult than the standard chorioallantoic method, and that positive and negative findings are easily differentiated. The only real difficulty concerns the interpretation of embryonic deaths. When the inoculation is properly carried out and the embryos are initially healthy very few non-specific deaths occur, and in any case doubtful results can be readily checked by suitable controls, sterility tests and subinoculation.

Discussion of other theoretical and practical aspects of the method will be deferred to subsequent papers.

SUMMARY.

The technique of amniotic inoculation of chick embryos is described.

All the influenza strains tested produce easily recognizable lesions when inoculated by this method, with the exception of the fully-adapted "Melbourne egg" strain.

[2] Seven successful isolations of influenza virus from human throat washings have been made by the amniotic method since this paper was submitted for publication.

The lesions affect primarily the epithelial lining of the respiratory tract, particularly within the developing lung.

Recognition of the fact of infection is based in practice mainly on the appearance of the cells in smears of fluid taken from the trachea.

After infection, large amounts of virus are present in both amniotic and allantoic fluids. The lung contains much larger amounts than any other organ.

REFERENCES.

Burnet, F. M. (1933): J. Path. Bact., 37, p. 107.
Burnet, F. M. (1936): Spec. Rep. Ser. Med. Res. Coun., Lond., No. 220.
Burnet, F. M. (1940): Brit. J. exp. Path., 21, p. 47.
Burnet, F. M. and Lush, D. (1940): Austral. J. exp. Biol., 18, p. 49.
Buddingh, G. J. and Polk, A. C. (1939): J. exp. Med., 70, p. 485.
Francis, T. and Magill, T. P. (1937): Proc. Soc. exp. Biol. and Med., 36, p. 143.
Francis, T. and Stuart-Harris, C. H. (1938): J. exp. Med., 68, p. 789.

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POLIOMYELITIS

4. THE SPREAD OF POLIOMYELITIS VIRUS IN CYNOMOLGUS MONKEYS WITH PARTICULAR REFERENCE TO INFECTION BY THE PHARYNGEAL-INTESTINAL ROUTE.

by F. M. BURNET AND A. V. JACKSON[1]

(From the Walter and Eliza Hall Institute, Melbourne).

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In previous papers (Burnet, Jackson and Robertson, 1939; Burnet, 1940) we described the susceptibility of *Macaca irus* (*M. cynomolgus*) to infection with poliomyelitis by the oral route, using a strain "Mar" isolated during the Victorian-Tasmanian epidemic of 1937. In this paper we have collected data, obtained over a period of three years, on the route by which the virus reaches the central nervous system after oral administration.

Technique. Two strains of virus were used in previous work; the Rockefeller Institute mixed virus "M.V." and the locally isolated strain "Mar," were both used in the form of spinal-cord from typically paralysed animals. Stock "M.V." virus has usually been derived from rhesus monkeys, "Mar" always from cynomolgus. Material was kept in glycerol-Ringer solution in a refrigerator.

Inoculations by the oral route were made by rubbing a thick emulsion of spinal-cord, ground with quartz powder and nutrient broth, over the tonsillar region with a swab of cotton wool on a suitably bent wire. The monkey was lightly anaesthetized, and after the swabbing was completed about 1 c.c. of emulsion was dropped into the pharynx. Intradermal inoculations were made into the skin of the upper arm; about 1 c.c. of 10 p.c. suspension was inoculated in a number of blebs.

In the experiments involving tests for virus in peripheral nerves, lymph nodes, etc., the paralysed animal was bled-out under anaesthesia and the various tissues obtained aseptically before exposure of the central nervous system. The nerves were placed in small volumes of glycerol-Ringer and stored in the refrigerator. After being drained they were minced with scissors and ground with quartz powder and broth; about 1 c.c. was used for small nerves, more for lymph nodes, etc. The emulsion was centrifuged, and 0·5 c.c. of the supernatant fluid used for intracerebral inoculations.

The pharyngeal tissue-extracts used in some experiments were made from a block of tissues including the tonsils, back of the pharynx and part of the soft palate and base of the tongue. As much muscle as possible was cut away and the remainder minced and finely ground with 7 or 8 c.c. of broth. The emulsion was centrifuged and the supernatant fluid shaken with 5 c.c. of anaesthetic ether and stored in the refrigerator overnight. The aqueous layer was removed and, after being tested for sterility, was used for inoculation. In the first experiment 0·25 c.c. was given intracerebrally in addition to 1 c.c. intradermally and 5 c.c. intraperitoneally; the animal showed severe immediate symptoms, became completely collapsed and took about an hour to recover from the injection. In other experiments, only intradermal and intraperitoneal inoculations were made.

As described previously (Burnet, Jackson and Robertson, 1939) some cynomolgus monkeys die after inoculation without showing definite premonitory symptoms. None of the deaths have been recorded as specific unless section of the spinal cord showed typical lesions, including perivascular cuffing, destruction of anterior-horn cells and areas of cellular infiltration.

Results.

1. *Infection after intradermal inoculation.* The investigation of strain "Mar" for its power to produce paralysis after intradermal inoculation was suggested by the finding of Trask and Paul (1938), that recently isolated strains infected by this route more readily than monkey-passage strains. Five "human" strains (less than three monkey passages) produced paralysis in five out of eight attempts. Seven "passage" strains (more than four monkey passages) were infective only twice in forty attempts. One strain "Wfd", which they regarded as particularly dermotropic, was examined at intervals between the third and sixteenth passages. Between the third and seventh passage it was infective six times out of ten, between the eighth and sixteenth only four out of seventeen attempts were successful.

[1] Work carried out under a grant for poliomyelitis research from the National Health and Medical Research Council.

Our first experiments showed that cynomolgus monkeys were readily infected by intradermal inoculation of strain "Mar", even in the form of the original human cord material. In view of the known difficulty of infecting rhesus monkeys with strain M.V. by this route, an attempt was made to determine whether the strain of virus or the different species of monkey was responsible for the difference. Table 1 combines the results of twenty-nine experiments.

TABLE 1.

Infection After Intradermal Inoculation of Poliomyelitis Virus.

Virus strain	Monkey.	Number inoculated.	Number paralysed.
"Mar" (first to ninth passages)	Cynomolgus	9	6 (67%)
	Rhesus	14	5 (36%)
"M.V."	Cynomolgus	6	0

It is evident that the ability to pass to the central nervous system from the skin, and to induce paralysis is shown by "Mar" in both species; the higher proportion of positive results in cynomolgus is probably only another manifestation of the greater susceptibility of this species by any route of inoculation.

2. *The centrifugal spread of virus in monkeys infected with poliomyelitis.* There are very few records of the isolation of poliomyelitis virus from the peripheral nerves of paralysed monkeys. Nicolau, Nicolau and Galloway (1929) claimed two successes out of six following intracerebral inoculation of peripheral nerve. Their criteria for successful inoculation, however, are unconvincing. One of the two monkeys showed no symptoms of poliomyelitis, but survived a later inoculation with virulent virus, whilst the other monkey developed only a transient paralysis of one arm which completely recovered. As the virus they were using was stated to be fully virulent and to produce regularly fatal paralysis, their results can be more readily interpreted as showing that passage-virus can be demonstrated only with difficulty, if at all in peripheral nerves.

We have found that with the strain "Mar" in cynomolgus monkeys many positive results may be obtained when peripheral nerves from animals infected by various routes are tested. Table 2 gives the results of such experiments in which infection had been induced by intracerebral or intraocular inoculation. The positive results were obtained only in cynomolgus monkeys, four positive results from two animals with "Mar", one with "M.V."

TABLE 2.

Presence of Virus in Peripheral Nerves After Infection by Intracerebral or Intra-ocular Routes.

			Result of Inoculating.		
Strain.	Species of Monkey.	Day Prostrate.	Vagus.	Sympathetic.	Sciatic.
"Mar"	Rhesus	16			0
	"	8	0		0
"M.V."	Rhesus	7	0		0
	"				
"Mar"	Cynomolgus	7	0	0	0
	"	8	++5	++6	+8
	"	8		0	0,0
	"	9	0	++8	0
"M.V."	Cynomolgus	6	0,0		++9,±
	"	6	0		0
	"	16	0		0

\+ Typical paralysis on day shown. ± Significant temperature rise only.
++ Typical paralysis with positive section. 0 No symptoms.

The other experiments on virus content of nerves were made with animals which had been inoculated peripherally and in these it is possible that the presence of virus in certain nerves resulted from passage from the periphery. In view of Sabin's (1937) findings with pseudorabies virus infections in rabbits, it seems likely that at least some of them represented spread from the central nervous system. These results will be noted in the sections dealing with intraperitoneal and oral inoculations.

3. *Intraperitoneal inoculation of "Mar" in cynomolgus monkeys.* Six cynomolgus monkeys were inoculated intraperitoneally with 2 c.c. of 10 p.c. virus suspension. Three were typically infected; two showed no symptoms but were immune to subsequent intracerebral injection of "Mar"; one showed no symptoms and died of intercurrent disease before being tested for immunity. Details are given in Table 3.

M. 657 showed definite tremor and rise of temperature seven to eight days after its test intracerebral inoculation but developed no paralysis. This is the only cynomolgus in our experience which has ever failed to die as a result of infection with poliomyelitis virus.

TABLE 3.

Results of Intraperitoneal Inoculation of Mar in Cynomolgus Monkeys.

Monkey.	Result.	Immunity test (intracerebral).
640	++7d	
641	0	
648	++7d	
641 (re-test)	0	0
656	++7d	
657	0	±7–8d
658	0	

The abdominal sympathetic chain was tested from M. 648 and produced typical paralysis in 10 days with positive histological findings. M. 656 showed paralysis involving first the upper part of the body. Virus was demonstrated in the vagus (cervical and thoracic) and in the thoracic sympathetic including the stellate ganglion; both produced typical tremor, paralysis and prostration in eight and seven days respectively. The abdominal sympathetic chain was non-infective.

4. *Infectivity of various tissues after oral infection.* We have previously shown that cynomolgus monkeys may be infected by the pharyngeal-gastrointestinal route (Burnet, Jackson and Robertson, 1939). The following experiments with a different shipment of monkeys show that the susceptibility of the species to infection by this route is a constant characteristic. Table 4 gives the essential details.

TABLE 4.

Results of Oral Administration of "Mar" Virus in Cynomolgus Monkeys.

Monkey.	Symptoms.	Histological findings.
709	104.4°F. Tremor 7 d. Killed	Lumbar typical cervical negative
710	Nil	
711	Incomplete paralysis 13 d. Killed	Lumbar and cervical typical
710 (Repeat)	Paralysis 11 d	Cervical typical lumbar negative
717	Nil	
718	Prostrate 9 d	Cervical and lumbar typical
719	104.0°F. excitable, early paralysis 10 d	Cervical and lumbar typical

The following notes on the symptomatology may have some bearing on the histological findings and on the distribution of virus in the body. M. 709 showed early weakness in the legs only, when killed. M. 711 had no premonitory signs and showed ptosis and general weakness in all limbs. M. 710 showed no symptoms in the first experiment and was tested again one month later. On the eleventh day it was grossly ataxic, with weakness most evident in the left arm and in the neck and back muscles of that side. It salivated profusely; the tongue was partially paralysed and deviated to the left. Movements of the eyes and jaws were normal. Histologically the medulla showed gross changes, the lumbar cord none. M. 718 was found prostrate on the ninth day without premonitory signs and showing only generalized weakness. It was thought not to be specifically infected and only a mesenteric lymph node was taken for test of virus content. Section of the cord was, however, typical of poliomyelitis. M. 719 showed completely typical symptoms of early paralysis with major weakness in the left leg.

In addition to material from these monkeys, tests had been made previously of mesenteric lymph node and certain nerves from a monkey, M. 275, which had been infected by laparotomy and direct injection into the lumen of the small bowel. This animal was briefly mentioned in our earlier paper (Burnet, Jackson and Robertson, 1939). It showed typical paralysis involving the right arm and neck muscles.

Tests for virus content of nerves and lymph nodes from these animals were made by intracerebral inoculation in cynomolgus monkeys. The results are shown in Table 5.

TABLE 5.

Distribution of Virus in Monkeys Infected by Oral or Intestinal Route.

Tissue.	M 709	M 710	M 711	M 718	M 719	M 275
Vagus	0	++6d	0			++9d, +13d
Sympathetic	++9d	++7d	0,0		±6–8d	0,0
Coeliac plexus etc.	++23d				0	0
Mesenteric lymph node		++6d*	+15d	++9d	0,0	+12d
Deep cervical lymph node		0			0	
Pharyngeal tissues		++7d	++8d		++6d	

*See text for further tests on these mesenteric lymph nodes.

In view of the theoretical importance of the isolation of virus from non-nervous tissue, further tests were made with mesenteric lymph nodes from M. 710. A pool of two slightly enlarged nodes in 5 c.c. of broth had been used for the original test. The same suspension was retested diluted 1: 10 and 1: 100; in addition a new suspension was prepared from another lymph node preserved in glycerol-Ringer. The 1: 10 dilution produced paralysis on the ninth day with prostration next day and typical changes in the cervical cord section. The monkey given 1: 100 suspension showed general weakness on the seventeenth day going on to prostration; the cord section showed typical gross lesions. The new suspension produced a rise of temperature on the fifth day with typical paralysis on the sixth and the characteristic appearance on section. In this animal, therefore, at least two mesenteric lymph nodes contained relatively large amounts of virus. In all cases the lymph nodes were carefully shelled out from between the layers of mesentery and could have contained no nervous tissue beyond the few nerve fibres stated to accompany the blood vessels into the nodes.

5. *Possible multiplication of "Mar" in human foetal tissues.* We include here a single unconfirmed experiment carried out in collaboration with Miss D. Lush owing to its bearing on the experimental results with cynomolgus monkeys. A human embryo of about twelve weeks was received from the Women's Hospital, Melbourne, after its removal by hysterotomy. Lung, intestine and buccal tissues (tongue, pharynx and soft palate) were removed separately to Tyrode solution. A portion of each tissue was used to set up Rivers-type tissue-cultures (5 c.c. of Tyrode solution plus minced tissue in each flask). 0·05 c.c. of a lightly spun 5 p.c. suspension of "Mar" virus was added and the cultures incubated for three days. The remainder of each tissue which had been stored in Tyrode solution in the refrigerator was then used to prepare a second set of cultures to which 0·5 c.c. of the first set was added. After three days incubation the lung culture was found to be contaminated with bacteria; the other two were sterile. The fluids were centrifuged. That from the lung culture was filtered through a gradocol membrane (A. P. D. 0·8μ) before inoculation; the other two were inoculated intracerebrally as untreated supernatant fluid. The monkey inoculated with the culture of mixed buccal tissues showed a high temperature (to 105.9°F.) on the sixth and seventh days and was prostrate on the ninth. The cord sections were typical, the lumbar being more heavily affected than the cervical region. The monkey given the intestinal tissue culture was found dead on the ninth day. It had shown a slight rise of temperature above its normal low level on the sixth and seventh days. Both cervical and lumbar cord sections showed typical lesions. The filtered lung culture gave rise to no symptoms.

We have been unable to obtain any other suitable human embryos to repeat this experiment, so that its implications must be accepted with great reserve. It is probable from our previous work that the original virus-suspension used would have infected at a dilution of 1: 1,000 but probably not beyond this. The material inoculated represented a 1: 1,000 dilution of the initial virus and, taking into account the fact that the infection was initiated in six days (to the first temperature rise), we feel confident that multiplication had occurred. It is obvious, however, that a single experiment is insufficient to establish this impression.

DISCUSSION.

It is still uncertain how the virus of poliomyelitis passes from one individual to another and in a susceptible subject eventually reaches the anterior-horn cells of the spinal cord. To solve these problems directly by clinical observation and laboratory tests of human material is difficult. Interpretations of the epidemiology and pathogenesis of the human disease have therefore been influenced by the findings of laboratory workers on the experimental disease in monkeys. With few exceptions the species used for experiment was the rhesus monkey (*Macaca mulatta*). In this animal poliomyelitis appears to be a disease strictly confined to nervous tissues, and the only "natural" route of infection is by the olfactory nerves. The widely accepted formulation by Faber (1934) of the pathogenesis of the human disease was based essentially on the work of Fairbrother and Hurst and others who used rhesus monkeys.

The view that human poliomyelitis is wholly a disease of the nervous system has never been acceptable to clinicians or epidemiologists, and the last two years have seen a tendency to look for primary infection in non-nervous tissues. Three groups of American workers (Harmon, 1937; Trask, Vignec and Paul, 1938; Kramer, Hoskwith and Grossman, 1939) have found that virus can be readily isolated from the faeces, particularly of non-paralytic cases. In Trask, Vignec and Paul's case the virus was isolated 2, 11 and 25 days after the onset, while Lepine and Sedallian (1939) report its isolation from the faeces 41 days after apparent

abortive poliomyelitis. On two occasions Paul, Trask and Gard (1940) isolated virus from sewage in cities where poliomyelitis was epidemic. These results suggest that at least in non-paralytic cases large quantities of virus pass into the intestinal contents, and that multiplication of the virus takes place in some tissue closely related to the intestinal tract.

In this and a preceding paper (Burnet, Jackson and Robertson, 1939) we have described the behaviour of a recently isolated poliomyelitis virus (strain "Mar") in cynomolgus monkeys. Comparative work with a standard passage strain "M.V." and with rhesus monkeys suggests that the results obtained with "Mar" in cynomolgus monkeys offer a more accurate parallel with the human disease than is found with any other combination of virus-strain and susceptible species.

The ease with which infection follows intradermal injection recalls the cases reported by Leake (1934), in which children inoculated with partially inactivated virus developed the disease. In view of the recent European and American discovery of virus in human faeces, a more important feature of the work with cynomolgus monkeys is their susceptibility to oral infection with the strain "Mar".

Our findings confirm those of Levaditi, Kling and Lepine (1929). The susceptibility of *M. irus* to oral infection with some strains is now fully established, and it remains to determine by what route virus reaches the central nervous system. Our chief results are: (1) that virus can be obtained consistently from the circumpharyngeal tissues; (2) that mesenteric lymph nodes almost always contain virus, sometimes in very high concentration; and (3) that portions of the autonomic nervous system frequently contain detectable virus.

The chief difficulty in interpreting these findings is to know whether virus in a given situation has reached this point from the periphery (in this case from the alimentary tract) or by centrifugal spread from the central nervous system. Centrifugal spread along nerves undoubtedly occurs in some animals inoculated centrally, but from our limited experience, this is exceptional. The rather large proportion of positive findings in the cervical-thoracic vagus and the sympathetic trunks is suggestive that it is along various autonomic nerve pathways that the virus reaches the central nervous system. Perhaps the least equivocal of our findings is the presence of virus in mesenteric lymph nodes—in one animal at least 100 minimal infective doses were present in an emulsion of lymph node. This cannot be explained by centrifugal spread along nerves. Multiplication within the lymph node seems very unlikely, so that we must conclude that virus multiplies in and is liberated from some tissue within the field of drainage of the mesenteric nodes, either the intestinal epithelium or the associated nervous elements.

Isolation of virus from the pharyngeal tissues has no special significance for cynomolgus infections as several authors have shown that virus may be almost constantly isolated from this region in rhesus monkeys infected intracerebrally. The fact that virus was obtained from the pharynx of M. 719 which was killed at an early stage when paralysis was just commencing suggests, however, that multiplication in the pharyngeal region was taking place.

The simplest interpretation of the experimental facts is that oral administration by the method used is followed by infection of the superficial pharyngeal tissues and of some portion of the intestinal wall. Virus then passes by the autonomic nerves to the central nervous system, frequently reaching the medulla and cervical cord before the lumbar region (as in M.710 and in two of the monkeys described in our previous paper). At the same time virus from the intestinal wall passes by the ordinary lymph channels to the mesenteric lymph nodes.

It seems reasonable to believe that the process by which virus becomes implanted in susceptible children and then passes to the central nervous system is of the same general character. However, the disease in cynomolgus monkeys is by no means identical with human poliomyelitis. We have never succeeded in producing contact infections, nor have we obtained evidence of the occurrence of subclinical immunizing infections by the natural route. Unsuccessful oral inoculation does not produce immunity; M.710 in Table 4 appears to have been completely susceptible to the second inoculation. Several other monkeys which resisted oral infection have been used subsequently for intracerebral inoculation of virus with posi-

tive results. The only cynomolgus monkeys which in our experience have developed substantial immunity after any method of inoculation are the two survivors (M.641 and 657) after intraperitoneal inoculation. One was wholly resistant to intracerebral inoculation, the other showed a definite temperature reaction but recovered without paralysis. No specific attempts to induce immunity by intradermal inoculation have been made, but practically all the animals which failed to become infected after one large inoculation were eventually tested by intracerebral inoculation and showed no immunity.

The single experiment in which apparent multiplication of our strain "Mar" took place in pharyngeal and intestinal tissues from a human foetus is described only as presumptive evidence that one strain at least of poliomyelitis virus is capable of infecting non-nervous tissues. If the experiment were confirmed the case for the application of the findings in cynomolgus monkeys to human pathology would be greatly strengthened.

SUMMARY.

Using the strain of poliomyelitis virus "Mar", infection of the central nervous system has been produced by intradermal inoculation in six of nine cynomolgus monkeys and in five of 14 rhesus monkeys.

After intracerebral infection virus can be isolated from peripheral nerves in some cynomolgus monkeys.

Infection may be produced in cynomolgus monkeys by intraperitoneal inoculation. In two instances such injections induced a high grade of immunity.

A further series of five typical infections following oral administration of virus strain "Mar" in cynomolgus monkeys is described.

The results of tests for virus in nerves, lymph nodes, etc., from these animals suggests that primary multiplication occurs in the pharynx and in the intestine, and that spread to the central nervous system takes place by the autonomic nerves.

In a single experiment multiplication of strain "Mar" probably occurred in tissue-cultures of pharyngeal tissues and of intestine from a human foetus.

REFERENCES.

Burnet, F. M., Jackson, A. V. and Robertson, E. G. (1939): Austral. J. exp. Biol., 17, p. 375.
Burnet, F. M. (1940): Med. J. Austral., 1, p. 325.
Faber, H. K. (1933): Medicine, 12, p. 83.
Fairbrother, R. W. and Hurst, E. W. (1930): J. Path. Bact., 33, p. 17.
Harmon, P. H. (1937): J. Amer. med. Assoc., 109, p. 1061.
Kling, C., Levaditi, C. and Lepine, P. (1929): Bull. Acad. Med., 102, p. 158.
Kramer, S. D., Hoskwith, B. and Grossman, L. H. (1939): J. exp. Med., 69, p. 49.
Leake, J. P. (1935): J. Amer. med. Assoc., 105, p. 2152.
Lepine, P. and Sadallian, P. (1939): C. R. Acad. Sci., 208, p. 129.
Nicolau, S., Nicolau, O. D. and Galloway, I. A. (1929): Ann. Inst. Past., 43, p. 1.
Paul, J. R., Trask, J. D. and Culotta, C. S. (1939): Science, 90, p. 258.
Paul, J. R., Trask, J. D. and Gard, S. (1940): J. Bact., 39, p. 63.
Sabin, A. B. (1937): Amer. J. Path., 13, p. 615.
Trask, J. D., and Paul, J. R. (1938): Science, 87, p. 44.
Trask, J. D., Vignec, A. J. and Paul, J. R. (1938): J. Amer. med. Assoc., 111, p. 6.

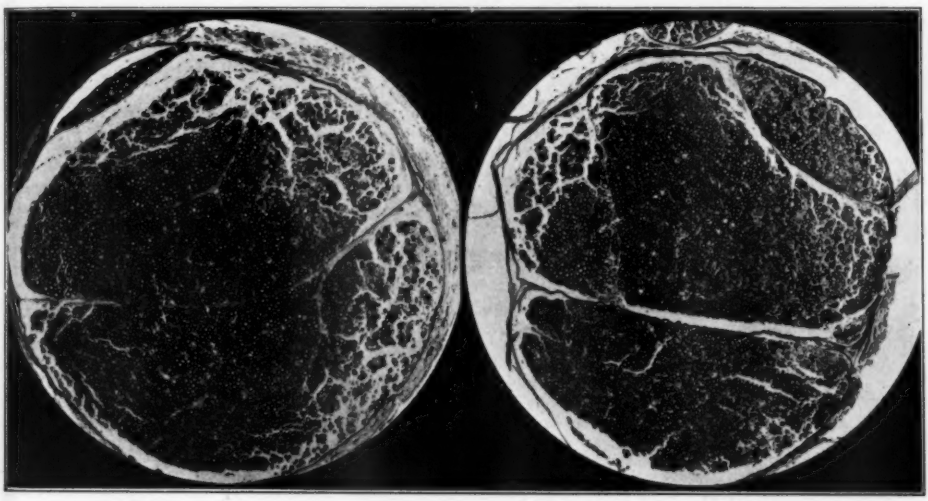
A NOTE ON THE DISTRIBUTION OF MYELINATED NERVE FIBRES WITHIN NERVE BUNDLES

by BASIL KILVINGTON

(From the Walter and Eliza Hall Institute, Melbourne).

(Submitted for publication 8th August, 1940.)

In the course of a nerve from the spinal root to the periphery, rearrangements of fibres take place in three main positions: (1) in the nerve-plexus proximal to the formation of the main nerve, as for example in the brachial or lumbo-sacral plexus, (2) in the nerve-bundles in the main trunks peripheral to the plexus, and (3) within the nerve-bundles themselves. Langley and Hashimoto (1917) have demonstrated (2) in detail in the nerves of the lower limb in the cat and in man, by micro-dissection after treatment with acetic acid to soften the connective-tissue between the bundles. This type of rearrangement, with some variation, is of general occurrence in mammals, anastomosis taking place between groups of bundles and single bundles. In the human perineo-sciatic nerve there are three areas where the rearrangement takes place, and no bundle proximal to a plexus corresponds even approximately with one distal to it.



Figs. 1, 2. Transverse section of the right ulnar nerve of a cat 1 cm. (Fig. 1) and 3 cm. (Fig. 2) below the level of partial division of the nerve 10 days previously.

The experiments reported here provide evidence for the third form of rearrangement, namely, within the nerve-bundles themselves. The method used was to study the course of degenerated fibres after transverse section of from ¼ to ½ of a peripheral nerve. The experiments were performed in cats, and the nerves used were the median and ulnar. Proximal to its division into many branches the former nerve consists of two or three bundles and the latter of a single bundle. Ten days after the section the animals were killed and the nerves dissected out, stained in 1 p.c. osmic acid, embedded in paraffin and sectioned transversely. Sections were examined immediately distal to the experimental lesion and at intervals of 1 cm. peripherally for 5 or 6 cm. In the most proximal section the degenerate fibres were found grouped near the surface in that part of the circumference of the nerve corresponding to the region of division, but in successive sections (Figs. 1, 2) the degenerate fibres were progressively more scattered throughout the section. Finally where the nerve was breaking up into its

terminal branches (Fig. 3) there were usually degenerate fibres in all the branches into which it divided. In some experiments one or more terminal branches were devoid of degenerate fibres.

In other experiments the greater part of the nerve was cut through and the distribution of the normal fibres followed. These occupied a single portion of the nerve immediately below the level of the section, but were increasingly scattered in distribution in successive sections towards the periphery.

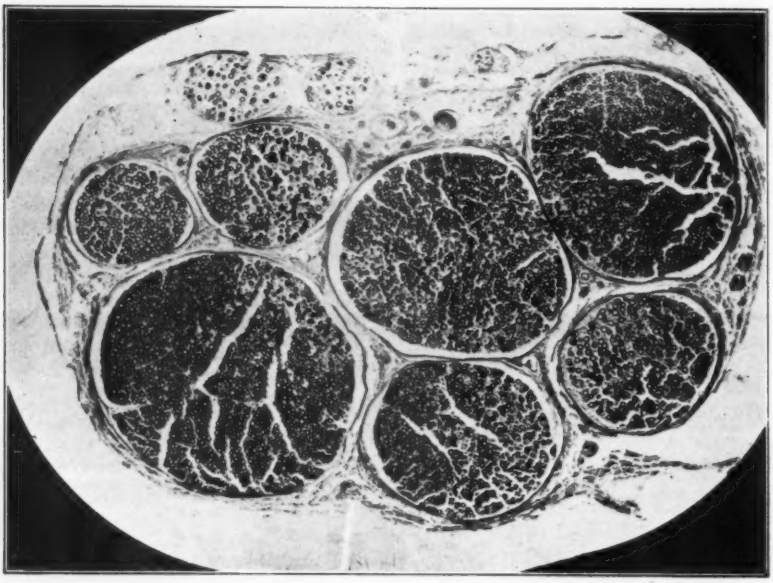


Fig. 3. Transverse section of the nerve 4 cm. distal to the experimental lesion, showing division of the nerve into terminal branches.

The rearrangement of nerve fibres in nerves explains two important clinical generalizations: (1) To produce complete paralysis of any muscle, pathological lesions in the spinal cord, such as those of infantile paralysis, have to be very extensive because a terminal motor or sensory branch not only possesses a widespread origin within single roots but receives fibres from several neighbouring roots.

(2) Unless it occurs immediately proximal to the origin of the distributing branch concerned, partial division of a nerve does not cause complete paralysis but only weakness of a muscle supplied by it, or in the case of a sensory twig leads only to a loss of the so-called epicritic sensation in the area of distribution.

REFERENCE.

Langley, J. N. and Hashimoto, M. (1917): J. Physiol. 51, p. 318.

CONCERNING GLYCOGEN BREAKDOWN IN THE LIVER

by P. FANTL, CHARLOTTE M. ANDERSON[1], AND J. F. NELSON
(From the Baker Institute of Medical Research, Alfred Hospital, Melbourne), Australia).

(Submitted for publication 20th August, 1940.)

PART I.

Until recently it was generally assumed that the breakdown of glycogen to glucose in the liver was due to a diastatic enzyme. However, since the investigations of Parnas and Baranowski (1935) and Cori and co-workers (1936), this question has had to be considered from a new aspect. Their findings necessitate a revision of our ideas concerning amylase activity, particularly as to what degree it contributes to glycogen breakdown or whether it has any real physiological significance. In support of the latter it must be emphasized that most workers who have studied amylase activity (Carruthers, 1935; Hollander, 1934; Hodgson, 1936; Glock, 1936) have used liver extracts or dried liver preparations. In this connection mention must be made of the work of Willstätter and Rohdewald (1934a), in which it was shown that tissues contain two different types of amylase, a lyo-enzyme which is soluble in the cellular fluid, and a desmo-enzyme which is bound to the protoplasm and liberated only after cellular disintegration. The object of the investigations described in this first section has been to ascertain whether amylase has a true physiological action, and studies have been made not only on tissue pulps but also on the intact liver.

Methods. Material: It appeared from the results of other workers that the rabbit was the most suitable animal for studying the breakdown of glycogen, because in this species the liver is supposed to contain no maltase. Wild rabbits have been used, and they were killed by a blow on the back of the neck and then bled from the carotid artery. In some instances the animals were bled under amytal anaesthesia, since stunning results in splanchnic stimulation and hence a high initial concentration of liver glucose. This procedure had no significant influence on the course of the reaction. The liver was removed as quickly as possible, one half being placed in a Petri dish and incubated at 37° C. The other half was minced through a fine bronze gauze and the pulp incubated at 37° C. At intervals of 10 minutes, samples were taken from both portions for chemical analyses.

Glycogen was estimated after digestion with 30 p.c. KOH by the technique of Good, Kramer and Somogyi (1933). Several other investigators of this problem have made glycogen determinations in a trichloracetic acid filtrate. We consider that this is an unsound procedure, as we have found that in this method up to 30 p.c. less glycogen is extracted than by KOH digestion. Willstätter and Rohdewald (1934b) have shown that only a portion of the glycogen can be extracted with solvents such as boiling water and trichloracetic acid.

In addition to glycogen estimations, a sample was taken for the estimation of reducing substances, and for this the liver samples, approximately 1 gm., were delivered into weighed tubes which were rapidly re-weighed and placed in the boiling water-bath. The proteins were then precipitated, whilst the tube remained in the water-bath, by the addition of 10 ml. of 0·45 p.c. $ZnSO_4$ followed by 2 ml. of N/10 NaOH. The tube was heated for five minutes, during which time the contents were ground up with a glass rod. After filtration and washing the contents were made up to the required volume and aliquots taken for the subsequent estimations. The reducing power of this solution was estimated by the method of Shaffer and Somogyi as modified by Harding and Downs (1933).

Glucose and Maltose: Differential determinations were carried out by the yeast fermentation method of Somogyi (1937), and checks were frequently made with freshly-prepared solutions containing both sugars. The estimation of glucose offers no difficulties, and in the determination of maltose the only interfering substance we have found in the present circumstances is glucose-6-phosphate. In the following experiments, with no phosphate buffer added, the initial inorganic phosphate concentration was approximately 30 mg. p.c. P, rising at the end of the incubation period to about 100 mg. p.c. At such low concentrations liver phosphatase is not inhibited, so we would not expect any accumulation of glucose-6-phosphate.

Results. The findings in six experiments on the lines described above are shown in Table I.

[1] Working under a full-time grant from the National Health and Medical Research Council,

TABLE 1.

Experiments 1–3: rabbits killed under amytal anaesthesia; Experiments 4–6: rabbits killed by a blow on the head. Samples taken at 10-minute intervals.

Expt. No.	Initial glycogen content of liver p.c.	Glucose mg. p.c. Whole liver.	Glucose mg. p.c. Liver pulp.	Maltose mg. p.c. Whole liver.	Maltose mg. p.c. Liver pulp.	Non-fermentable reducing substances expressed as ml. N/200 $Na_2S_2O_3$ per gm. tissue. Whole liver.	Non-fermentable reducing substances expressed as ml. N/200 $Na_2S_2O_3$ per gm. tissue. Liver pulp.
1	2·8	530	690	0	35	7·8	8·0
		950	1,100	0	92		
		1,300	1,500	0	74		
		1,300	1,880	0	102		
		1,200	2,100	0	129		
		1,450	2,200	0	119	7·8	8·0
	Glycogen p.c. Liver pulp.						
2	2·6	480	390	0	86	4·4	3·6
	1·95	1,000	1,000	0	138		
	1·7	1,250	1,300	0	127		
	1·45	1,300	1,400	0			
	1·3	1,250	1,500	0	132		
	1·25	1,300	1,700	0	120	4·7	4·2
	Initial glycogen p.c.						
3	0·5	380	340	21	58	5·5	2·9
		790	750	37	67		
		790	770	27	110		
		780	790	0	126		
		730	810	0	136		2·9
		710				6·6	
4	0·43	353	314	0	72	6·9	3·6
		510	532	25	90		
		617	600	0	126		
		673	837	0	112		3·3
		636		49			
		582		38		5·5	
5	1·1	357	364	0	0	5·7	2·5
		970	900	0	107		
		930	1,070	67	59		
		1,040	1,200	48	104		
		900	1,100	0	143	5·2	2·6
		1,030	1,200		202		
6	0·9	465	463	0	0	6·6	3·4
		860	1,000	0	50		
		1,150	1,250	0	59		
		980	1,300	0	93		
		880	1,450	0	41		
		950	1,400	0	53	5·1	4·0

From Table 1 it can be seen that in experiments Nos. 1, 2 and 6 with whole liver no maltose could be detected during the incubation period of 50 minutes. In the three other cases maltose, in amounts ranging from 21 to 67 mg. per 100 gm. of tissue was found, whereas maltose was always present in the liver pulp in concentrations ranging from 40 to 202 mg. per 100 gm. of tissue. However, in all instances the values for maltose represent, on the average, less than 10 p.c. of the values found for glucose. No attempt was made to eliminate the influence of blood diastase, as some investigators have done, by first perfusing the liver with saline or Ringer's solution. Even admitting that the liver was not free from blood, the results indicate that amylase plays a minor rôle in minced liver and does not exert any significant effect on whole liver. The figures for the increase of non-fermentable reducing substances, in the few cases when this did occur, are too small to have any significance in the breakdown of glycogen.

The graphical recording (Fig. 1) of two experiments clearly shows the ratio between maltose and glucose formation. The course of the reaction does not give a regular curve because, for the sake of comparison between the intact and the pulped liver, buffer solution could not be added, and by the action of phosphatase inorganic phosphate would be set free, thus altering the pH. The figures, however, do indicate that most of the glycogen is converted into glucose, and from

Table 1 it can be seen that the loss of glycogen and the increase in fermentable sugar are, within the limits of experimental error, equal, indicating that no appreciable amounts of other products have been formed, e.g. dextrins, hexose-phosphates or lactic acid.

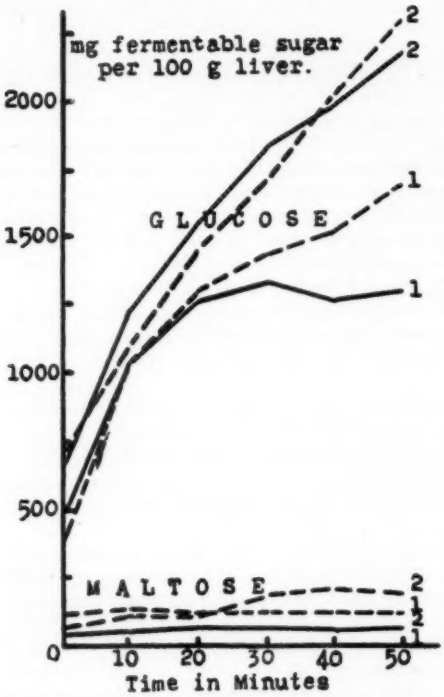


Fig. 1. Ground liver, dotted line. Whole liver, solid line.

Although to some extent the experimental errors with whole liver may be greater than with a more homogeneous liver pulp, the results definitely indicate that the undamaged liver cells show no amylase activity. However, after destruction of the cell structure an enzyme system converting glycogen into maltose becomes apparent, but this effect is of minor significance when compared to the other findings.

As a further control to show that no maltase enzyme exists in the rabbit's liver, known amounts of maltose were added to freshly-ground liver and the usual estimations carried out. These results are shown in Table 2.

TABLE 2.

30 gm. liver pulp + 35 mg. maltose dissolved in 10 c.c. M/15 Sörensen's phosphate buffer pH 7·2. Incubated at 37° C. Figures given for 100 gm. of tissue.

Time in min.	Glycogen p.c.	Glucose mg. p.c.	Maltose mg. p.c.
0	4·0	250	150
20		915	210
70		1,200	280
90		1,200	310
150	2·7	1,320	330

The figures recorded indicate that rabbit's liver does not contain maltase.

Three experiments were also carried out that showed maltase activity to be absent from rabbit's serum. Glycogen was added to the sera to give a concentration of 300 mg. p.c. After incubation at 37° C. for four hours, the glucose concentration was found to be unaltered and in addition, whilst no maltose was initially detected, a final value of 142 mg. p.c. was found,

Three other samples of sera were incubated with maltose for four hours at 37° C. and at the end of this time the glucose and maltose concentrations were unchanged. Control experiments with dog's serum showed that added glycogen or maltose was converted to glucose.

These findings indicate that in these investigations it was not necessary to take any special precautions to obtain a liver free from blood, since any glucose formation could not have been due to blood enzymes.

PART II.

The experiments just discussed, whilst not elucidating the enzyme system that is responsible for the breakdown of glycogen in the liver, do show that in the rabbit's liver the formation of glucose from glycogen is certainly not due to an amylase plus maltase activity. Parnas *et al.* (1935) have found that inorganic phosphate is concerned in the mechanism. They consider that a glucose-monophosphate is the first step of the degradation to glucose, and Cori and co-workers (1938) and Kiessling (1938) identified the first degradation product as glucose-1-phosphate (Cori ester) which is converted by a second enzyme system, the so-called phosphoglucomutase, into glucose-6-phosphate (Robison ester). Each of these compounds is, according to the findings of Cori and co-workers (1938), split by the liver phosphatase into dextrose and inorganic phosphate. All of the above workers' experiments were carried out with fresh tissue extracts (liver, muscle, brain, heart, etc.) as well as with the purified and phosphatase-free enzyme preparations from liver (Cori, 1939).

These results indicated that glycogen is broken down only in the presence of inorganic phosphate with adenylic acid as co-enzyme or rather, as it does not act as a phosphate carrier (Kiessling, 1939), as an activator, and that a corresponding amount of glycogen is found in the form of glucose phosphate. It would appear that either glucose-1 or glucose-6-phosphate is the precursor of dextrose and therefore of blood sugar.

Ostern, Herbert and Holmes (1939) claim that liver pulp incubated with inorganic phosphate and M/200 NaF, the latter added supposedly to inhibit the phosphatase activity, gives as sole end-product, glucose-6-phosphate; whereas Lee and Richter (1940) state that the phosphorylation in liver pulp was always much less than the total glycogenolysis. No difference was observed between pulps from perfused and non-perfused livers.

In this section of the work experiments have been carried out to investigate the influence of varying concentrations of phosphate and fluoride on glycogen breakdown.

Methods. The material used was freshly-prepared liver pulp to which an equal volume of Sörensen's phosphate buffer pH7·2 was added. To one half of the mixture NaF was added in amounts shown in the protocols of the individual experiments. The reaction mixture was incubated in air at 37° C. and samples for chemical analyses taken at intervals of one and two hours.

Inorganic phosphate (directly estimable) was determined in a trichloracetic acid filtrate after appropriate dilution by the method of Kuttner and Cohen (1927). Total phosphate was determined in the trichloracetic acid filtrate after ignition with 10 N H_2SO_4 and perhydrol. Glucose and NaF in varying concentrations were without influence on the colour development. Reducing and fermentable substances were determined as in the previous section. In experiments with M/4 phosphate, deproteinization was carried out by the addition of 20 ml. 0·45 p.c. $ZnSO_4$ and 2 ml. N/10 NaOH.

Inconsistent results were obtained with livers from animals killed under amytal anaesthesia. In several instances the total acid-soluble phosphorus increased during incubation. A similar observation was made with liver pulp to which veronal buffer was added. These barbituric acid derivatives apparently affect the breakdown of trichloracetic-acid-insoluble phosphoric acid compounds, and so the use of these compounds was discontinued and the animals killed by a blow on the head. The addition of phosphate and NaF did not change the total acid-soluble phosphorus during the incubation period.

To determine the relative formation of glucose-1 and glucose-6-phosphate, the Ba salts were prepared from the reaction mixture by the method of Cori and Cori (1931). From the initial reduction of the solution containing the purified Ba salts and the reduction after hydrolysis for 10 minutes in N H_2SO_4, the amounts of the two hexose phosphates could be calculated. The results were checked by the determination of readily-hydrolysable phosphorus and total phosphorus in the Ba salts.

Results. In Table 3 the results of seven experiments with varying concentrations of phosphate and NaF are shown. It will be observed that: (1) Up to concentrations of M/30 phosphate in the reaction mixture no influence of phosphate on the glycogen breakdown is apparent. Either with M/200 NaF or without it (Experiment 1) the sole end-product of glycogen breakdown is glucose.

(2) Higher concentrations of inorganic phosphate in the reaction mixture, from M/30 to M/4 (Experiments 2–7), bring about a significant change in the breakdown of glycogen, since the reaction products now consist of hexose-monophosphate and glucose, in amounts which balance the loss of glycogen. However, it will be noted that even with high concentrations of phosphate the

TABLE 3.

Formation of glucose and hexose–monophosphate (H.M.P.) in liver pulp. Apart from initial liver glycogen content, all values are given in milligrams per 100 grams of liver tissue.

No.	Initial Glycogen gm. p.c.		Time of incubation hours	Glycogen disappearance calculated as glucose A	Glucose formed B	H.M.P. formed calculated as glucose C	Total of B + C	Alteration in directly estimable phosphate mg. p.c. P	Percentage glycogen accounted for. $\frac{B+C}{A} \times 100$	Composition of reaction mixture.
1	6·1	(a)	1	2,240	2,380	0		+14	106	Phosphate M/45 + M/200 NaF
			2	2,650	2,800	0		+72	105	
		(b)	1	2,020	2,230	0		+16	110	Phosphate M/45 Without NaF
			2	2,450	2,600	0		+60	106	
2	5·0	(a)	1	1,920	1,700	625 (P)	2,325	−108	120	Phosphate M/30 + M/10 NaF
			2	3,150	2,900	650 (P)	3,550	−112	116	
		(b)	1	2,700	2,460	0		+24	90	Phosphate M/30 Without NaF
			2	3,050	2,900	0		+72	95	
3	5·2	(a)	1	2,350	2,200	384 (P)	2,584	−66	110	Phosphate M/30 + M/5 NaF
			2	3,400	3,400	75(Ba)	3,475	−58	102	
		(b)	1	2,950	2,800	0		+12	95	Phosphate M/30 Without NaF
			2	3,400	3,200	60(Ba)	3,260	+52	96	
4	4·2	(a)	1	3,370	2,560	900 (P)	3,460	−156	103	Phosphate M/10 + M/10 NaF
			2	4,020	4,120	240(Ba)	4,340	+60	107	
		(b)	1	3,950	3,150	490 (P)	3,640	−84	92	Phosphate M/10 Without NaF
			2	4,200	4,050	150(Ba)	4,200	+44	97	
5	6·0	(a)	1	2,900	2,150	580 (P)	2,730	−100	94	Phosphate M/10 + M/10 NaF
			2	4,600	3,750	460(Ba)	4,210	−60	92	
		(b)	1	4,250	3,500	600 (P)	4,100	−104	97	Phosphate M/10 Without NaF
			2	5,800	5,300	300(Ba)	5,600	−82	97	
6	6·1	(a)	1	2,750	1,670	1,600 (P)	3,270	−280	119	Phosphate M/4 + M/10 NaF
			2	5,240	4,270	1,700(Ba)	5,970	−344	114	
		(b)	1	4,040	2,100	1,400 (P)	3,500	−232	87	Phosphate M/4 Without NaF
			2	6,030	4,300	1,620(Ba)	5,920	−256	98	
7	Fasting rabbit. Glycogen added conc. 3·2 p.c.	(a)	Min. 35	2,070	1,720	308(Ba)	2,030		98	Phosphate M/7·5 + M/200 NaF
		(b)	35	2,040	1,760	290(Ba)	2,050		101	Phosphate M/7·5 Without NaF

(P) = Hexose phosphate calculated from decrease in inorganic phosphate.
(Ba) = Hexose phosphate determined from barium salts.

major part of the glycogen disappearing is found as glucose and the minor part as glucose phosphate. This fact is puzzling since Martland and Robison (1927) have shown that bone phosphatase in the presence of concentrations of M/10 phosphate cannot hydrolyse glycero-phosphoric esters. Thus it must be assumed that either the liver phosphatases are not completely inhibited, even in concentrations of M/4 phosphate and additional M/10 NaF, or that they are not necessarily concerned in the mechanism with which we are now concerned. In this case glucose-1 and 6-phosphates would have to be regarded not as intermediate but rather as stabilization products.

(3) Addition of M/200 NaF to M/45 or stronger phosphate solutions does not inhibit the phosphatase activity, as judged by the ratio of glucose and glucose phosphate formation. Higher fluoride concentrations added to the phosphate solution have a demonstrable effect on the phosphatase activity (Expts. 2, 3, 4 and 5) especially in the first hour of incubation. The addition of M/10 or M/5 NaF to the phosphate solution (Expts. 2, 3, 4 and 6) causes a greater phosphorylation than in the experiments without NaF. Hexose-monophosphate, calculated from the decrease in inorganic phosphate, shows that, apart from the formation of glucose-monophosphate, some other not directly estimable phosphate compound has been formed.

At first it was thought that hexose-di-phosphate was formed, a reaction which has been described by Kalckar (1939) as occurring in kidney extracts to which glucose and NaF were added under aerobic conditions. However, in an experiment in which one part of liver pulp prepared from a fasting rabbit and one volume of a solution containing 1 p.c. glucose in M/2 phosphate buffer and M/5 NaF were incubated for an hour, no decrease of glucose took place, whilst a small decrease of inorganic phosphorus occurred. A similar decrease of inorganic phosphorus was observed in another experiment without addition of glucose. Thus it appears that the alterations of inorganic phosphate in the presence of NaF are not alone due to the formation of hexose-phosphate.

In our experiments with fresh pulp it is most probable that both acid and alkali phosphatases were present, whereas in autolysed extracts the alkaline form would most likely be in a greater concentration, and this may explain the contradictory results obtained by different workers concerning the influence of NaF on phosphatase activity.

Experimental recordings in Table 4 show the influence of the incubation time on the ratio of glucose–1 to 6–phosphate in liver pulp and muscle extracts. It will be seen that after a short incubation period (15 minutes) glucose–1–phosphate preponderates. In spite of the high concentration of inorganic phosphate the phospho-glucomutase which converts glucose–1–phosphate to the 6–isomer is still active. Indeed, we find that with muscle extracts incubated under the same conditions as in Table 4 for a longer period (30 minutes) the isolated Ba salts consist of 85 p.c. glucose–6 and only 15 p.c. of glucose–1–phosphate. The same is true for liver. The hexose–phosphate fraction in the experiments 2–7 (2 hours' incubation), recorded in Table 3, consisted almost entirely of glucose–6–phosphate.

TABLE 4.

Formation of glucose monophosphates in liver and muscle during short periods of incubation.

Liver: The reaction mixture consisted of one part of minced liver and two volumes of M/2 phosphate buffer pH 7·2 containing M/5 NaF. Incubated at 37° C. and equal parts removed at 0, 5, 10 and 15 minutes.

Time in minutes.	Glucose–6–PO_4 formed.[2] mg. per 100 gm. liver.	Glucose–1–PO_4 formed. mg. per 100 gm. liver.
0	103	47·5
5	112	134
10	126	178
15	144	274

Muscle Extract: Phosphate extract prepared and poisoned with iodo–acetic acid after Kiessling (1938). Samples taken as with liver.

Time in minutes.	Glucose–6–PO_4 formed. mg. per 100 gm. muscle.	Glucose–1–PO_4 formed. mg. per 100 gm. muscle.
0	8·6	41·5
5	28·5	220
10	40·0	260
15	69·5	350

As previously mentioned, glucose values were obtained after a yeast fermentation and the criticism may be made that the hexose–phosphates might under our conditions also be fermented. However, it was found that neither glucose–1–phosphate, prepared from rabbit muscle extract after poisoning with iodoacetic acid by the method of Kiessling (1938), nor glucose–6–phosphate, obtained by substantially the same process but after a longer incubation time, showed any

[2] Although the term glucose–6–PO_4 is used for convenience, it should be noted that the transformation products of glucose–1–phosphate are a mixture of glucose–, fructose– and mannose–6–phosphate.

fermentation in half an hour in an alkaline solution. Under these conditions glucose is completely fermented. Fermentation in neutral solution for 2½ hours, as is necessary for complete fermentation of maltose, did not break down glucose–1–phosphate, but 60 p.c. of the glucose–6–phosphate disappeared. (See Table 5.)

TABLE 5.

Fermentation of hexose–monophosphates with baker's yeast.

	Initial value. mg. p.c.	After 30 min. incubation with yeast in 0.1 p.c. Na_2CO_3.	After 2½ hrs. incubation with yeast in neutral solution.
Glucose–6–phosphate calculated as glucose	38·2	38·2	15·6
Glucose–1–phosphate calculated as glucose (after hydrolysis with N/H_2SO_4 for 10 min.)	24·0	24·8	22·3

The fact that the glucose–monophosphates are fermented very slowly is remarkable since these compounds could be isolated by Kiessling (1939) and Schäffner (1939) from yeast preparations. A more rapid fermentation should be expected if they are activated glucose derivatives. It must be pointed out in this connection that Neuberg and Kobel (1926) have already recorded the slow fermentation of Robison ester in comparison with the free sugars and this occurs, even in cell-free press juice of yeast, where the permeability would not play any rôle. Meyerhof (1935) also mentions that hexose–di–phosphate (Harden and Young ester) is not itself the intermediate product of rapid glycolysis, but a kind of stabilization product which ferments relatively slowly.

It is also noteworthy that while glucose–6–phosphate ferments more readily than glucose–1–phosphate, the latter is easily hydrolyzed by weak acids, whereas glucose–6–phosphate is relatively stable.

DISCUSSION.

Some recent investigations of Somogyi (1940) are of considerable significance in the interpretation of the results recorded in this paper. In a detailed study on diastase activity Somogyi emphasizes the fact that besides reducing dextrins and maltose, glucose is a normal product of diastatic activity. However, it is shown that, although the relative amounts of the different intermediates may vary according to the enzyme or substrate concentration, the non-fermentable reducing substances (dextrins) and maltose form the bulk of the reaction products. In comparison with these, glucose formation is small and does not occur in the early stages of the incubation period.

These facts, applied to our results with intact rabbit's liver, namely, that at any time in the incubation period of 50 minutes the major portion of the reaction products was glucose, and that frequently non-fermentable reducing substances and maltose were hardly estimable, do not support the theory that amylase activity is responsible for dextrose formation. Further evidence is gained from the experiments with minced liver where, although small amounts of maltose could regularly be detected, the main end-product was again glucose.

These latter findings lend weight to the assumption by Willstätter and Rohdewald (1934a) of the existence of a desmo-amylase, which is bound to the living cell and only exercises its activity after cellular disintegration.

The influence of inorganic phosphate on the glycogen breakdown is, in a qualitative respect, in accord with the observations of other workers, namely, that increasing phosphate concentrations allow a greater glycogen breakdown.

The inorganic phosphate which disappears is found in corresponding amounts of hexose-monophosphate. A short incubation period, say, 15 minutes, produces a mixture of hexose-monophosphates which is richer in glucose-1-phosphate than glucose-6-phosphate, whereas with a longer incubation period the glucose-1-phosphate originally formed is changed into the 6-isomer by the action of phosphoglucomutase. This transformation may occur even in the presence of high inorganic phosphate concentration, and is also observed in muscle extract which has been poisoned with iodoacetic acid in order to eliminate the oxido-reduction

system which would convert the hexose-monophosphate into fructose-di-phosphate (Harden and Young ester). Extension of the incubation time over 15 minutes decreases the yield of glucose-1-phosphate in favour of the 6-phosphate, and in fact we have used this observation for the preparation of glucose-6-phosphate.

Concerning the influence of sodium fluoride on glycogen breakdown, in contrast to Ostern *et al.*, we find that a concentration of M/200 NaF has no inhibitory influence on the phosphatase, and in the presence of weak phosphate concentrations the sole end-product is still glucose. Stronger fluoride concentrations, M/10 or M/5, have a definite effect on the phosphatase activity, but the influence of NaF is not confined to a phosphatase inhibition because the phosphorus uptake in the presence of NaF is apparently due to the formation of substances other than hexose-monophosphates. Thus calculations made from the inorganic phosphate decrease would indicate that more inorganic phosphate disappears than could be accounted for by the formation of hexose-monophosphate. Furthermore, the greater uptake of inorganic phosphorus in the presence of NaF is not likely to be due to the formation of hexose-di-phosphate, since we were unable to find any phosphorylation of glucose added to liver pulp.

So far our observations are in agreement with those of other workers, but if we consider the balance as found by us between glycogen disappearance and glucose-monophosphate formation, it will be apparent that glucose-phosphate represents only a portion of the glycogen which disappears. The greater part of it is found as glucose. In one experiment with the relatively high concentration of M/4 phosphate, the ratio of the glucose-monophosphate to glucose was still 1 : 1·5. If the breakdown of glycogen goes through glucose-phosphate, a phosphatase, according to Cori *et al.*, must act on this to produce dextrose. The equation

$$\text{Phosphate } \alpha \text{ glucoside} \rightleftharpoons \alpha\text{-glucose} + \text{phosphoric acid}$$

under the influence of liver phosphatase should be a reversible reaction, which in high concentration of inorganic phosphate should be shifted to the left, and if the concentration of inorganic phosphate is high enough, no formation of glucose should be expected. Actually it was found that M/10 sodium fluoride, which inhibits the activity of acid phosphatase when combined with M/4 phosphate, could not inhibit the formation of glucose. Since we have shown that, in the rabbit liver, the glucose formation cannot be due to an amylase activity, we must assume that either liver phosphatase is not inhibited by high phosphate concentrations and additional sodium fluoride or that glucose phosphates are stabilization products of the true intermediates.

Glucose added to liver pulp could not be phosphorylated. Thus the hexose-monophosphates, it might be said, cannot be secondary reaction products. If we assume that hexose-phosphates are not really the intermediates, but that inorganic phosphate is still necessary for the breakdown, then the inorganic phosphate would play the rôle of a co-enzyme.

SUMMARY.

The sole end-product of glycogen breakdown in unperfused intact rabbit's liver is glucose.

In unbuffered liver pulp glucose forms the bulk of the breakdown product, while an average of 10 p.c. of maltose is found.

The blood content of rabbit's liver cannot have a significant influence on glucose formation since rabbit's serum does not contain maltase.

Pulped rabbit's liver, mixed with phosphate buffer, converts glycogen partly to glucose-monophosphate and partly to glucose.

Even with concentrations of M/4 phosphate, more glucose is formed than glucose-phosphate.

Short incubation times (15 minutes) give mixtures richer in glucose-1 than glucose-6-phosphate. With longer incubation times only glucose-6-phosphate can be detected.

Liver phosphatase cannot be inhibited by M/200 NaF in M/45 or M/7·5 phosphate buffer. M/10 NaF has a definite inhibitory influence on phosphatase activity, especially in the first hour of incubation. M/4 phosphate and additional M/10 NaF still produce glucose and glucose-phosphates in nearly the same amounts.

The decrease of inorganic phosphate in liver pulp containing NaF is not only due to glucose-monophosphate formation.

Glucose-1-phosphate (Cori ester) is not fermented by baker's yeast under conditions in which glucose and maltose are completely fermented. Glucose-6-phosphate (Embden-Robison ester) is also not fermented by baker's yeast under conditions in which glucose is completely fermented, but 60 p.c. is fermented under conditions in which 100 p.c. maltose is fermented. A possible explanation of these observations is discussed.

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REFERENCES.

Carruthers, A. (1935): J. biol. Chem., 108, p. 535.
Cori, G. T. and Cori, C. F. (1931): J. biol. Chem., 94, p. 561.
Cori, C. F. and Cori, G. T. (1936): Proc. Soc. exp. Biol. & Med., 34, p. 702.
Cori, C. F. and Cori, G. T. (1937): Proc. Soc. exp. Biol. & Med., 36, p. 119.
Cori, G. T., Colowich, S. P. and Cori, C. F. (1938): J. biol. Chem., 123, p. 375.
Cori, G. T., Cori, C. F. and Schmidt, G. (1939): J. biol. Chem., 129, p. 629.
Glock, G. E. (1936): Biochem. J., 30 (2), p. 2313.
Good, C. A., Kramer, M. and Somogyi, M. (1933): J. biol. Chem., 100, p. 485.
Harding, V. J. and Downs, C. E. (1933): J. biol. Chem., 101, p. 487.
Hodgson, T. H. (1936): Biochem. J., 30 (1), p. 542.
Hollander, L. (1934): Science, 79, p. 17.
Kalckar, H. (1939): Biochem. J., 33, p. 631.
Kiessling, W. (1938): Biochem. Z., 298, p. 421.
Kiessling, W. (1939): Biochem. Z., 302, p. 50.
Kiessling, W. (1939): Naturwissenschaften, 27, p. 129.
Kuttner, T. and Cohen, H. R. (1927): J. biol. Chem., 75, p. 517.
Lee, M. and Richter, D. (1940): Biochem. J., 34, pp. 353 and 551.
Martland, M. and Robison, R. (1927): Biochem. J., 21, p. 665.
Meyerhof, O. (1935): Helv. Chim. Acta, 18, p. 1030.
Neuberg, C. and Kobel, M. (1926): Biochem. Z., 179, p. 451.
Ostern, P., Herbert, D. and Holmes, E. (1939): Biochem. J., 33, p. 1858.
Parnas, J. K. and Baranowski, T. (1935): Compt. rend. Soc. Biol. Paris, 120, p. 307.
Schäffner, (1939): Naturwissenschaften, 27, p. 195.
Somogyi, M. (1937): J. biol. Chem., 119, p. 741.
Somogyi, M. (1940): J. biol. Chem., 134, p. 301.
Willstätter, R. and Rohdewald, M. (1934a): Z. physiol. Chem., 225, p. 103.
Willstätter, R. and Rohdewald, M. (1934b): Z. physiol. Chem., 229, p. 256.

THE INFLUENCE OF SULPHYDRYL COMPOUNDS UPON THE ACTION OF INSULIN

by A. B. CORKILL AND A. H. ENNOR[1]

(From the Baker Institute for Medical Research, Alfred Hospital, Melbourne, Australia).

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The fact that insulin may be inactivated by reduction of the disulphide linkages has long been recognized, and has been the subject of considerable research. Admittedly the effect of powerful reducing agents, apart from reduction of the disulphide linkages, is to damage the molecular structure of the compound, but in this investigation we were concerned only with that type of inactivation produced by mild reducing agents such as cysteine and reduced glutathione (GSH), a type of inactivation which may have some physiological significance in the organism. Allen and Murlin (1925) reported that crystalline insulin, inactivated by treatment with H_2S, could be reactivated by exposure to air; but these experiments have not received confirmation by other workers (Blatherwick *et al.*, 1927).

du Vigneaud *et al.* (1931-32) were probably the first to investigate the effect of cysteine and GSH upon insulin. They found that cysteine and GSH, when added to insulin in neutral or alkaline solution, produced a rapid inactivation. It was thought that the loss of activity of the insulin was due solely to the reduction of S-S linkages and not to fission of the insulin molecule, but experiments designed to re-oxidise the -SH groups did not result in reactivation of the insulin. Wintersteiner (1933), further investigating this problem, showed that the rate of inactivation by cysteine and GSH increased with increasing pH over the range pH 6–8. Similar results were also obtained using thioglycollic and α-thiolactic acids. This inactivation apparently can be reversed if special precautions are taken, as indicated by the work of Freudenberg and Wegmann (1935). These workers were able to regenerate up to 80 p.c. of reduced insulin by oxygenation in solution and treatment with peroxide. However, it was emphasized that in many instances the insulin molecule was so badly damaged that regeneration was impossible.

Jacobs (1939) extended these investigations to a study of the *in vivo* effect of cysteine upon the hypoglycaemic action of insulin. His results indicated that injected cysteine had a definite action in reducing the intensity and duration of the hypoglycaemic response to insulin. Levine *et al.* (1939) have sought to correlate the sulphydryl content of the tissues with insulin sensitivity, and state that animals which are known to be hypersensitive to insulin have decreased stores of GSH in the liver. Their experiments were carried out on rats, some of which had been hypophysectomized, some adrenalectomized, and others treated with phyone, a dried anterior pituitary preparation containing the growth hormone. The two former groups were shown to have markedly reduced GSH amounts in the liver, and animals which had undergone treatment with phyone showed slightly, but in the present authors' opinion, not significantly greater amounts of GSH. As far as blood and muscle GSH were concerned, little alteration was noticed and, having regard to the fact that GSH is possibly only one of the factors involved, these workers put forward the suggestion that the liver is more important for insulin inactivation than blood and other tissues.

Because some experimental data which we had available appeared to conflict with the supposition that GSH was responsible, in part, for the inactivation of insulin, we decided to carry out some further experiments with a view to ascertaining the importance of this mechanism in the intact animal. The procedure adopted by Levine and co-workers was (a) to produce insulin-sensitive states by hypophysectomy and adrenalectomy, and (b) to produce a lowered GSH content of the tissues

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by the administration of iodoacetic acid or alloxan. Their experiments with these latter substances, however, were unsuccessful; but in any case it appears to us that results obtained by the use of such substances would be of doubtful value because of the widespread disturbances in fundamental physiological processes known to follow their administration.

In our experiments we have used substances the injection of small amounts of which was not necessarily followed by marked physiological disturbances.

The experimental studies are divided into two portions, the first dealing with insulin resistant states and the second with conditions wherein the animal displays a hypersensitiveness to insulin.

Methods. English hutch rabbits were used throughout, and were maintained on a diet of cabbage leaves and bran with occasional carrot. GSH determinations were carried out with the method of Woodward and Fry (1932).

For the estimation of reducing substances 1 ml. of blood was taken from the marginal ear vein and the proteins precipitated by 9 ml. of Somogyi's $Zn(OH)_2$ solution (Friedemann and Graeser, 1933). Total reducing substances were determined in 1 ml. of filtrate by the method of Hagedorn and Jensen.

Another aliquot of 5 ml. was made slightly alkaline by the addition of a minute quantity of solid $NaHCO_3$; 5 ml. of a 10 p.c. washed yeast suspension were then added, and after 3–5 minutes in the water-bath at 37° C., the suspension was centrifuged and 5 ml. of centrifugate taken for the determination of residual, non-fermentable, reducing substances. All values shown in the Tables have been corrected and represent blood glucose only.

I. *Insulin-resistant Animals.*

(a) *Anterior pituitary extracts.* Cope and Marks (1934) have shown that injection of crude saline extracts of anterior pituitary into rabbits will produce an insulin-resistant condition. One of us (Ennor, 1939) has shown that extracts known to be active as regards their power to produce an insulin resistance in the rabbit also produced a diminished GSH content in the liver. Later these experiments were extended and the extracts shown to have a similar effect on rats.

The apparently contradictory nature of our own observations as compared with those of Levine *et al.* (1939) may possibly be due to our use of fresh glands whilst these workers used a dried gland preparation (phyone) containing chiefly the growth-promoting factor. In addition, experiments carried out here indicate that the factor which produces lowering of GSH content is destroyed on heating to 40–50° C. and moreover is lost unless the glands are stored in a frozen condition. It thus appears possible that the factor influencing GSH in liver would be absent from a preparation such as phyone.

Ennor's experiments have been confirmed by recent work of Gregory and Goss (1939), only an abstract of which is at present available to us. These workers, apparently using extracts containing a powerful growth hormone, were able to show a significant decrease in both liver and muscle GSH.

(b) *Well-fed rabbits.* Although the degree of sensitivity to insulin varies from animal to animal, it is obvious that, if well fed, an animal will exhibit a relatively greater power to resist the hypoglycaemic action of insulin than if starved.

(c) *Diphtheritic toxaemia.* The harmful effects of infection upon carbohydrate metabolism are well known and particularly well recognized in the treatment of the diabetic state. Even in the normal individual a toxaemia may result in decreased glucose tolerance and the appearance of a mild but temporary diabetic condition. One of us (Corkill, 1932), investigating the effects of a diphtheritic toxaemia upon the carbohydrate metabolism of young rabbits, has shown that a toxaemic condition results in a resistance to the effect of insulin. Generally this resistance is characterized by an early recovery from the hypoglycaemic effects of this hormone. Furthermore, it was also shown that the administration of insulin to these toxaemic animals did not result in a deposition of liver glycogen.

In the experiments in this section diphtheria toxin supplied by the Commonwealth Serum Laboratories was used and 4 M.L.D. were injected subcutaneously into the abdominal wall of young rabbits. As an early toxaemic effect was desired the animals were sacrificed 24 hours after injection, and their livers taken for determination of the GSH content. This early toxaemic condition, wherein no microscopic lesions exist in the adrenals or liver, and where the latter is still able readily to form glycogen from glucose, is one in which a definite insulin resistance is present; later stages are associated with widespread damage, and are to be avoided in studies of this nature. In all cases control animals receiving diphtheria toxin were injected with two units of insulin and the blood sugar response determined. The results of these tests need not be shown here, but they were indicative of quite a definite insulin resistance.

II. *Insulin-sensitive Animals.*

(a) *Adrenalectomy.* Levine and his associates, as previously mentioned, used rats throughout their experiments, but here, as in other sections of this work, our choice of experimental animal was the rabbit. This animal does not exhibit the well-known symptoms which are characteristic of adrenal insufficiency, and as far as we are aware, no explanation of this phenomenon has been advanced other than the obvious suggestion that the animals must have accessory cortical tissue. In all the animals used here we have been unable to detect any trace of accessory cortical tissue. For these examinations we are indebted to the Pathologist of the Alfred Hospital, Dr. R. A. Willis. However, since we were only concerned with the production of an insulin sensitivity, this aspect did not concern us. In fact, the rabbit would appear to be the best choice because the results obtained are not complicated by marked changes in carbohydrate and fat metabolism as well as circulatory failure.

Semi-adult rabbits were taken and adrenalectomized in a two-stage operation, the right suprarenal being first removed through the abdominal route, care being taken to avoid damage to the liver and inferior vena cava. After complete healing of the skin incision (about eight days) the left suprarenal was removed and the animals deprived of cabbage for 24 hours to avoid the onset of diarrhoea, which at this stage is always fatal. They were then placed on their usual diet until they were taken for experimental purposes, which was from 2–3 months after removal of the second adrenal. In all cases careful post mortem examinations were made for the presence of residual adrenal tissue and the results obtained only from those animals from which the adrenal tissue had been entirely removed are illustrated.

(b) *Ergotoxine.* The administration of ergotoxine in appropriate amounts abolishes the motor effects of adrenaline and sympathetic stimulation, and thus results in an increased sensitivity to the action of insulin. Accordingly the effects of this drug upon the GSH content of the liver were investigated. 5 mg. of ergotoxine (Ergotoxine ethanosulphonate—Burroughs, Wellcome & Co. Ltd.) were dissolved in a small amount of saline and injected intravenously. At the end of 1½ to 2 hours liver samples were taken in the usual manner for estimation of GSH content. This dosage of the drug, whilst producing an insulin sensitivity, did not result in any other noticeable physiological disturbances.

(c) *Starvation.* By reduction of the carbohydrate stores of the body a relative insulin sensitivity can be obtained, so much so that young rabbits, aged from 8 to 10 weeks, often exhibit spontaneous hypoglycaemia resulting in severe convulsions and death, following the deprivation of food for more than 16 hours. In these experiments young adult rabbits were placed in metabolism cages in a warmed room and fasted for 24 hours. At the end of this period liver samples were taken for analysis. The results of the experiments in these two sections are illustrated in Table 1.

TABLE 1.

GSH mg. p.c.

Insulin-resistant Animals.		
Treated with anterior pituitary extract.	Well fed.	Toxaemic.
177	300	300
264	320	270
182	365	268
182	312	405
139	310	330
Mean: 189	320	315

Insulin-sensitive Animals.		
Adrenalectomized.	Starved.	Injected with 5 mg. ergotoxine.
360	258	346
340	240	290
436	320	320
260	315	315
260	296	
Mean: 331	286	318

It is obvious that, apart from the animals receiving treatment with anterior pituitary extract, the GSH values show no significant variations. Furthermore, the values in pituitary-treated animals are low, and these animals are resistant to the hypoglycaemic action of insulin.

Experiments in vivo *with Cysteine and GSH.*

Here Jacobs' experiments, wherein cysteine was injected subcutaneously into rabbits, were repeated and extended by also using GSH. Cysteine was given in the same amounts

and over the same time intervals as used by Jacobs. Blood for sugar analyses was taken at half-hourly intervals following the intravenous injection of two units of insulin. One week was allowed to elapse before the experiments were repeated using cysteine in addition to insulin. The results are illustrated in Table 2.

TABLE 2.

True Blood Glucose, mg. p.c.

R_1		R_2		R_3		R_4	
Insulin	Insulin + Cysteine	Insulin	Insulin + Cysteine	Insulin	Insulin + Cysteine	Insulin	Insulin + Cysteine
106	117	119	110	119	113	112	103
Insulin, 2 units ⟶							
70	92	68	74	76	71	59	100
56	77	59	72	87	82	52	78
56	74	52	79	71	106	67	83
56	77	56	81	75	134	79	105
79	79	72	97	84	138	93	114
				97	131	96	121

From the results shown here it would appear that the treatment with cysteine has had a definite effect in reducing the intensity of the hypoglycaemic response to insulin. However, the increase in the blood sugar in rabbits 3 and 4 following the injection of cysteine and insulin led us to suspect an action of cysteine per se upon the blood sugar level. Jacobs reported some increases in the blood sugar following cysteine, but apparently did not regard them as being of significance. It seemed, however, that such increases might account for the apparent diminution of insulin action, and accordingly control experiments with cysteine were performed. Three rabbits, fasted for 24 hours, were used, No. 1 being a semi-adult and Nos. 2 and 3 fully grown. Cysteine, 0·6 gm. per kg. body weight, was injected subcutaneously in two divided doses. The initial dose contained two-thirds of the total amount, and the remainder was given twenty minutes later.

In rabbit No. 1 blood sugar determinations were made at 45-minute intervals, and in Nos. 2 and 3 at 30-minute intervals. To check chance fluctuations two fasting blood sugars were taken at the usual time intervals.

TABLE 3.

Blood Glucose following Subcutaneous Administration of Cysteine.

	Blood Glucose—mg. p.c.		
Rabbit No.	1	2	3
Fasting (1)	94	134	124
" (2)	90	134	127
Cysteine ⟶			
	136	152	163
	159	154	168
	177	184	175
		202	224

Such marked increases would certainly mask the hypoglycaemic effect of insulin, and hence we cannot regard the results shown in Table 2 as being due solely to inhibition of insulin action by cysteine.

All the experimental work so far described was carried out on the intact animal in which there is a continual absorption of food from the intestine, and in addition there is the somewhat uncertain influence of the various endocrine glands. This, of course, will tend to render the interpretation of the results more difficult, and in an endeavour to simplify conditions the eviscerated spinal cat was used. This may be regarded as a muscle preparation with its own intact blood supply, and in which the injection of insulin causes a loss of blood glucose which is either oxidized or stored as muscle glycogen.

If glucose is infused into the external jugular vein at an appropriate rate, it is possible to maintain the blood sugar at a constant level, and furthermore the injection of insulin will produce decreases that are comparable from animal to animal. Such a preparation is therefore most suitable for determining whether cysteine exerts *in vivo* a direct inhibitory effect on the hypoglycaemic action of insulin. The results of several experiments on these lines are shown in Table 4.

TABLE 4.

The Influence of Cysteine and GSH on Insulin Action in the Spinal Eviscerated Cat.

Intravenous infusion of glucose at rate of 0·12 gm. per kg. per hour.

Control Animals. True blood glucose—mg. p.c. 2 hrs.				Test Animals. True blood glucose—mg. p.c. 2 hrs.			
Cat Wt. kg.	Initial.	after 10 units insulin.	Decrease mg. p.c. per hr.	Cat Wt. kg.	Initial.	after 10 units insulin.	Decrease mg. p.c. per hr.
3·5	331	176	78	(a) 3	274	59	107
3·7	318	173	72	(b) 5	282	170 (1 hr.)	112
2·8	210	50	80	(c) 2·8	217	50	84
3·2	298	147	76	(d) 2·4	278	124	77
3·4	327	183	72				
3·5	340	190	75				
3·3	202	92	55				

Animals (a), (b) and (c) received cysteine and animal (d) glutathione. Apart from animal (a), where the total quantity of cysteine given was 0·75 gm., the amount of sulphydryl compound injected was 1·5 gm. This was given in three divided doses.

In all cases except the animal weighing 5 kg. the amount of insulin administered was 10 units, and in the exception 15 units were given. All injections were made into the femoral vein, and blood for sugar analysis was taken from the carotid artery.

From the results shown in Table 4 it is apparent that no effect has been obtained by the administration of cysteine or GSH in so far as inhibition of hypoglycaemia is concerned. The magnitude of the fall of the blood sugar following insulin is very similar in both series of experiments, and is conclusive evidence that in such a preparation the effect of cysteine is not of the same nature as in the intact animal and no direct inhibition of insulin action is seen.

DISCUSSION.

From the experimental results which we have obtained there appears little basis for the assumption of Levine and co-workers that -SH compounds are of importance in the mechanism whereby insulin is inactivated in the animal body. Whilst we have been able to confirm the results of Jacobs in so far that cysteine appears to inhibit the hypoglycaemic action of insulin in the intact animal, we do not believe that this effect is due solely to a direct action between cysteine and insulin. As shown, the administration of cysteine is followed by increases in the blood glucose which can adequately account for the apparent diminution of insulin action. In addition to this it must be admitted that deductions based upon treatment with doses of a compound which are in vast excess of the amounts found in the entire animal are open to serious criticism.

In these experiments we have not been able to confirm the statement of Levine *et al.* that the GSH content of the livers of animals known to be hypersensitive to insulin is significantly lower than in normal animals. On the contrary animals receiving injections of a crude extract of the anterior pituitary gland and showing a definite insulin resistance have, in addition, a markedly lowered GSH content in the liver. In the light of our own experiments the recent work of Goadby and Richardson (1940) is very interesting. These workers have found that the rate of disappearance of injected insulin is not affected by exclusion of the liver from the circulation. On the other hand, they have been able to show that nephrectomized animals are unable to inactivate or remove insulin from the blood, and as a result there is a persistence of the insulin action. It is also of interest to note that these workers have been unable to detect any difference in the rate of disappearance of insulin from the blood of rabbits which have previously received injections of the glycotrophic factor of the anterior pituitary gland and thus have an insulin resistance.

SUMMARY.

The *in vivo* effect of cysteine and GSH upon the hypoglycaemic action of insulin and the relationship between liver GSH and insulin sensitivity have been studied.

We have been able to confirm the work of Jacobs in so far as the inhibitory effect of cysteine upon insulin action in the intact animal is concerned. However, experiments on the eviscerated animal have given no evidence of a direct inhibitory action by cysteine. It is very probable that the effect of cysteine in diminishing the action of insulin in the intact animal is due to the fact that cysteine *per se* causes a marked increase in blood glucose.

No relationship has been found to exist between GSH content of the liver and insulin sensitivity.

Acknowledgements. One of us (A.H.E.) wishes to express his thanks to the National Health and Medical Research Council, under whose grant this work was carried out. We are indebted to Dr. F. G. Morgan, Director of the Commonwealth Serum Laboratories, for supplies of diphtheria toxin.

REFERENCES.

Allen, R. S. and Murlin, J. R. (1925): Proc. Soc. exp. Biol. & Med., 22, p. 492.
Blatherwick, N. R., Bischoff, F., Maxwell, L. C., Berger, J. and Sahun, M. (1927): J. biol. Chem., 72, p. 57.
Cope, O. and Marks, H. P. (1934): J. Physiol., 83, p. 157.
Corkill, A. B. (1932): J. Physiol., 75, p. 381.
Ennor, A. H. (1939): Austral. J. exp. Biol., 17, p. 173.
Freudenberg, K. and Wegmann, T. (1935): Z. physiol. Chem., 233, p. 159.
Friedemann, T. E. and Graeser, J. B. (1933): J. biol. Chem., 100, p. 291.
Goadby, H. K. and Richardson, J. S. (1940): J. Physiol., 97, p. 417.
Gregory, P. W. and Goss, H. (1939): Growth, 3, p. 159. Abstracted in Biol. Abstracts, 14, p. 599, Ref. 6402, 1940.
Jacobs, H. R. (1938): Proc. Soc. exp. Biol. & Med., 38, p. 305.
Levine, R., Hechter, O., Groseman, A. and Soskin, S. (1939): Proc. Soc. exp. Biol. & Med., 40, p. 525.
du Vigneaud, V., Fitch, A., Pekarek, E. and Lockwood, W. W. (1931–32): J. biol. Chem., 94, p. 233.
Wintersteiner, O. (1933): J. biol. Chem., 102, p. 473.
Woodward, G. E. and Fry, E. G. (1932): J. biol. Chem., 97, p. 465.

NOTES ON THE LIFE HISTORY AND ARTIFICIAL BREEDING OF THE AUSTRALIAN "STICKFAST" FLEA, *ECHIDNOPHAGA MYRMECOBII* ROTHSCHILD

by M. W. MULES

(From the Animal Health Research Laboratory, Parkville, Victoria, Council for Scientific and Industrial Research, Division of Animal Health and Nutrition).

(Submitted for publication 29th August, 1940.)

The purpose of these experiments was to develop convenient methods for breeding and handling native "stickfast" fleas in the laboratory, the ultimate object being to use them in experiments to determine their capacity to act as vectors of *Virus myxomatosum*, a specific pathogen for the European rabbit.

Preliminary observations on the association of fleas with the wild introduced rabbit were made during the month of March, 1939, at Point Pearce Station, which is situated on Yorke Peninsula, South Australia. Many wild rabbits were found to be heavily parasitized with native "stickfast" fleas, and small lice were also present on all rabbits examined. Having determined that engorged fleas will live for a number of days after removal from their rabbit host, an attempt was made to transport them to Canberra, where preliminary breeding experiments were to be carried out. Approximately three hundred adult fleas were sent in rabbit fur in small vials, but only seventy of them survived the journey. They were placed on rats at Canberra, and a culture was started from the eggs they deposited. Of the two hundred and fifty-three fleas which died in the vials, two hundred and forty-seven were *Echidnophaga myrmecobii* and six were *Echidnophaga perilis* Jordan. A large series of specimens in alcohol from Point Pearce was examined and contained both species, *E. myrmecobii* predominating. *E. perilis* was present in the culture in the first and second generations of laboratory-bred fleas but was not present in the third, or later generations. The disappearance was accidental, and a study of this species has not been possible, but it is likely to be similar to that of *E. myrmecobii*.

LIFE HISTORY OF *E. MYRMECOBII*.

The egg. This is oval, perfectly smooth, pearl-white and semi-translucent, approximately 0·24 mm. in the smaller diameter. The egg shell is soft and is easily damaged.

Incubation period. At 22° C. this ranges from four to five days. The egg loses its pearl-white lustre after about three days, and starts to assume a light straw colour, which is most pronounced immediately before the larva hatches. Approximately 95 p.c. of all eggs incubated in these experiments have been fertile and have hatched out. Many eggs slightly dented during sieving or other handling have hatched satisfactorily.

The larva. This has a short spine on the head enabling it to rupture the egg shell which is split in a longitudinal direction around the end of the egg and half-way along each side. The freshly hatched larva is pearl-white and approximately 1·15 mm. long and 0·12 mm. in diameter. It wriggles out of the egg slowly (usually being clear of it within ten minutes). It starts to feed when from one to three hours old. A fully fed larva is approximately 3 mm. long and 0·37 mm. in diameter. The larvae are much less hairy than those of *Ctenocephalus canis* and *C. felis*. The first moult occurs on the third or fourth day, and a second one has been observed on the sixth or seventh day. Larvae reach maturity after feeding for from nine to fourteen days.

Larvae which had fed for seven days lived in sand without food for seven days; they were then supplied with food, started to feed again, and pupated after being in the larval stage for from fifteen to eighteen days. Freshly hatched larvae lived for four days without feeding.

TABLE 1.

Age of larva.	Average weight.	Age of larva.	Average weight.
1 day	0·025 mg.	6 days	0·131 mg.
2 days	0·039 „	7 „	0·166 „
3 „	0·039 „	8 „	0·196 „
4 „	0·053 „	9 „	0·197 „
5 „	0·093 „		

When given food they matured normally and had a normal pupal period. Incubation at 30°C. for twelve hours proved fatal to most larvae but a few lived for as long as twenty-four hours. Unless food is available to larvae until they are fully grown they are unable to pupate. Table 1 shows the average rate of growth of larvae fed on dried rabbit blood. Larvae were weighed daily in batches of twenty, and the average weight estimated.

Food for larvae. Larvae refused to feed on several grades of meatmeal and bonemeal, excreta from rats and rabbits, or on scurf, hair, etc., scraped from the skin of rats and rabbits. Dried ox or rabbit blood has been used as food in these experiments and was mixed with sand to provide a suitable culture medium. Dried citrated blood also proved a suitable food for larvae.

Other species of flea larvae have been recorded as feeding on the excreta of the adult flea. It seems probable that the excreta of the adult are also natural food of larvae of *E. myrmecobii.* In a single experiment it was found that 42 fleas of mixed sexes excreted 7·9 mg. faeces in 14 hours, which is equal to 0·013 mg. from one flea in one hour. Larvae fed on ox or rabbit blood grew to maturity as rapidly as those fed on the excreta of adult fleas. Sikes (1923) has shown that the larvae of *Ceratophyllus wickhami* can be successfully fed on dried mammalian blood.

The pre-pupal stage. All food is expelled from the gut approximately twenty-four hours before the larva begins to spin a cocoon. The grub becomes pure white and semi-translucent. At this stage it loses its active habits although it continues to travel about in the medium sluggishly searching for a suitable spot in which to spin a cocoon. The spinning larva assumes a U-shape within the cocoon and pupates three to four days later. If disturbed after making the cocoon and before it pupates, the larva usually leaves it and, apparently unable to make a second, eventually dies.

There is a tendency for larvae to pupate on the surface of the medium when fine sand is used, but in coarse sand the majority pupate beneath the surface. Dirt, fine sand and sawdust were found satisfactory to use in the media for breeding larvae, but clean sand has the advantage of being easily sieved and does not become lumpy when moistened.

The cocoon. This is oval, approximately 2·5 mm. long and 1·4 mm. in diameter. When pupation takes place in fine sand it is much smaller than when coarse sand, dirt or other debris is attached to the outside of it. The cocoon proper is composed of a loosely woven network of silken strands to the outside of which particles of sand or other foreign matter adhere. Some are usually fastened against the breeding container, permitting the pre-pupal and pupal stages to be observed through the glass. When freshly made, the cocoon is easily collapsed but becomes stronger as the silken material hardens. The delicate pupae are consequently well protected and can be sieved without harm from the sand or earth in which they have formed.

The pupa. Pupation takes place four to five days after the cocoon is completed. The pupa remains white for eight to nine days. After nine or ten days the colour of the pupa alters and takes on a slight brownish tint which becomes more pronounced each day until the adult is ready to emerge.

Emergence of adult. The cocoon is ruptured suddenly and the adult flea emerges and leaves it within two or three seconds. The adult emerges as an active insect and is ready to establish itself on the host immediately. Dissection of a number of nine- and ten-day old cocoons showed that adults were active within them prior to a normal emergence. Vibration or disturbance of cocoons containing mature adults causes many to emerge and they immediately become very active. This phenomenon suggests that in nature it is the habit of these fleas to wait within the shelter of their cocoons until such time as some disturbance indicating the presence of a possible host causes them to emerge suddenly.

The adult flea. The approximate size of freshly hatched unfed fleas has been found to be:

Male, 1·22 mm. long and 0·52 mm. in diameter. Female, 1·32 mm. long and 0·62 mm. in diameter.

The average size of engorged fleas after they have been feeding for seven days is approximately:

Male, 1·33 mm. long and 0·73 mm. in diameter. Female, 2·21 mm. long and 0·66 mm. in diameter.

After feeding for two or three days the female is easily distinguished, being larger than the male. If fertilized it continues to increase in size for a further four or five days. The male increases in size only slightly after becoming engorged. An engorged female is more sluggish than when unfed, and cannot travel about on the host as well as the male. The colour of adult fleas varies considerably, females varying from light brown to reddish brown, males from reddish brown to black.

Unfed fleas of both sexes have been found capable of jumping six and one half inches in a vertical direction and eight and one half inches horizontally. From a smooth horizontal surface they usually jump at an angle of about 60°.

Establishment on the host. An experiment to determine the method of attachment t

the host was carried out by placing fourteen unfed one-day old fleas on dry sand in a 3¼" × 14¼" beaker. They were allowed to settle down for half an hour. A young rat was then admitted to the beaker; the fleas immediately became very active and all fourteen gained a footing on the rat within one minute, each flea immediately travelling against the animal's fur towards the head. This habit has been observed on rabbits also. Two fleas were found attached to the rat after ten minutes, eight after a further twenty-five minutes, five more after one hour, and all fourteen were attached after one and a half hours, eight being fastened to the rat's ears and six around its eyelids and nose. The rat endeavoured to remove some fleas as they were attaching and succeeded in getting rid of three, but these regained the host and finally attached themselves. The fleas did not cause the rat any apparent inconvenience after they had become attached. An inflamed area was noticed on the rat's ear around each flea a short time after it had become attached.

Copulation. Copulation takes place on the host and has been observed among fleas three and a half days old which had been established on the host for three days. The act is performed whilst the female is attached to the host. In one instance a pair was joined for twenty-five minutes before the act finished.

Male fleas have been observed to detach and reattach themselves on a different part of the host. Some male fleas migrated to a new rabbit when it was placed in a small cage with a parasitized animal. Females rarely detach; although they have been seen to move to fresh positions on the host, this always took place before they became fully engorged.

Pre-oviposition period. Some female fleas were dissected to determine at what time copulation and oviposition took place. Eggs were present in the oviducts of a flea four and a half days old, which had fed on a rat for four days. Sperms were also present in the spermatheca. Fertile eggs were deposited by fleas five days old which had been feeding on the host for four and a half days with males of the same age.

Oviposition. Eggs deposited by the attached fleas drop off the host readily and do not tend to stick to the fur. The act of oviposition has been observed on numerous occasions and the female has projected the egg after passing it, as has been recorded by Parman (1923) in *E. gallinacea.* From four to six eggs are deposited daily during the early stages of oviposition, but fewer during the later stages. A single female is capable of depositing approximately two hundred and fifty eggs during its life.

Although the warmth of the host is transmitted to the fleas to some degree, oviposition has been stimulated during cold weather by raising the room temperature to about 22°C. Observations have shown that light or dark during the day and night has no effect on oviposition.

Length of life on the host. Males have been found on rabbits as long as three weeks after the animal was parasitized although most disappear towards the end of the third week. Females live longer, and have been found on rabbits up to 57 days after parasitization, the average life of the female being about 40 days. Spent fleas usually live for from one to three days in a semi-comatose condition after leaving the host.

In Tables 2 and 3 are shown examples of the survival rates of fleas on white rats.

TABLE 2.

Rat.	Dates of emergence of fleas.	No. of fleas.	Date of death of last flea.	Maximum life (Days)
1	20–21/4/39	15	31/5/39	41
2	22/4/39	11	16/6/39	55
3	23–24/4/39	21	14/6/39	52
4	27/4/39	11	6/6/39	40
5	? 27–28/4/39	No record	31/5/39	? 34
6	20–21/5/39	23	3/8/39	42

TABLE 3. Details of Survival on Rat 1.

Date.	No. of fleas present.	No. of days of life.	Date.	No. of fleas present.	No. of days of life.
21/4/39	15	—	19/5/39	8	29
10/5/39	15	20	20/5/39	8	30
11/5/39	14	21	21/5/39	7	31
12/5/39	14	22	22/5/39	5	32
15/5/39	12	25	23/5/39	3	33
16/5/39	11	26	24/5/39	1	34
17/5/39	9	27	31/5/39	1	41
18/5/39	9	28	2/6/39	0	—

Survival of unfed fleas. A number of pupae was left on the bottom of a deep Petri dish with a little dry sand at 22°C. Adults emerged over a period of four days. The lid of the dish was left in place and no moisture added at any time. Several fleas lived for eight and a half weeks and two for as long as seventeen weeks. In a further experiment several fleas were placed with dry sand in a glass tube plugged with cotton wool. They were kept at 26°C.

The majority was dead in six days, two survived for seventeen days, and one for twenty-one days.

The length of the life cycle. These observations can be summarized by stating that under laboratory conditions in an incubator at 22° C. the life cycle of the flea is complete in from 30 to 46 days. The periods in this cycle are as follows:

Pre-oviposition period	4– 5 days.
Egg incubation period	4– 5 days.
Larval period	10–14 days.
Pupal period	12–22 days.
Total time, egg to egg	30–46 days.

It is likely that the pupal period is much longer during winter months under natural conditions.

BREEDING *E. MYRMECOBII* UNDER LABORATORY CONDITIONS.

Method adopted to collect living fleas from rabbits. The fleas should be removed immediately after killing their rabbit host. Pieces of skin having fleas attached are removed with scissors, and are placed in a large securely corked bottle with a little dry earth which serves to take up the moisture. The pieces of skin are removed from the field bottle with long forceps, and the fleas shaken into a large smooth paper funnel which guides them into a clean container. Some fleas detach in the field bottle and are picked up on a piece of skin and shaken off in the funnel. Fleas that do not detach easily are often induced to do so if they are returned to the field bottle and shaken vigorously. Brushing fleas with a camel-hair brush serves to make them unfasten, but it is a tedious process. A small glass funnel is used to transfer fleas to small vials or other containers.

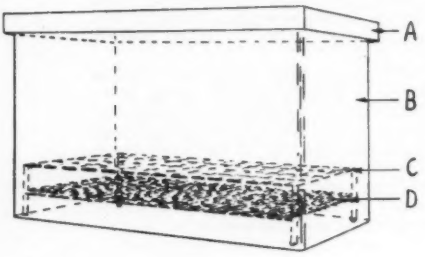

Fig. 1. Jar used to collect flea eggs.
(a) Cover.
(b) Glass battery jar.
(c) Removable wire mesh platform.
(d) Cloth egg-collecting tray.

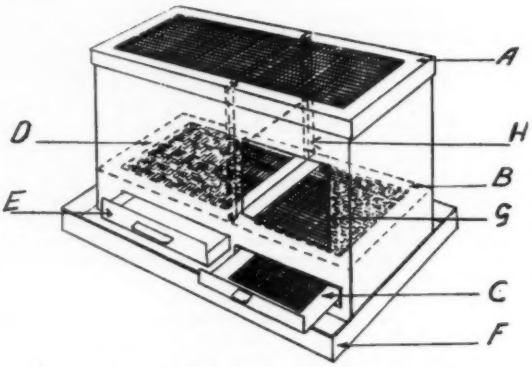

Fig. 2. Cage used for collecting flea eggs from rabbits.
(a) Cover.
(b) Egg-collecting compartment.
(c) Cloth egg-collecting tray.
(d) Feeding compartment.
(e) Cleaning tray.
(f) Tray providing oil moat.
(g) Glass inspection plate.
(h) Vertical sliding door.

Artificial breeding technique. In preliminary breeding experiments at Canberra, white rats were used as hosts. They proved suitable for producing small flea cultures and were used to rear the first, second, and third generations. Unless the rats were kept in separate containers they defleaed each other to some extent. Rats were parasitized with twenty to twenty-five fleas each. As it was necessary for other experiments that the fleas should be bred on rabbits, rabbit kittens were substituted as hosts. Wild rabbit kittens have been found capable of carrying one hundred to one hundred and fifty fleas without showing any ill effects. They have been used as hosts for as long as four weeks, when they grew too large for the jars used to house them.

Parasitizing rabbits and rats. Two methods have been used to establish freshly emerged adults on rats and rabbits. (a) Fleas placed in small glass tubes will attach themselves to

a rabbit's ear if the tube is placed against the bare area on the inside of the ear. Sometimes this method is tedious as it is necessary to hold the tube firmly against the ear while the fleas attach themselves. (b) Some sand is placed in a large glass jar with the rat or rabbit. The fleas are then placed in the jar, if possible on the head of the animal. Fleas that do not establish themselves immediately, or are brushed off, regain a footing on the animal if it is left in the jar for a short time. In cold weather it is necessary to warm the atmosphere to about 22°C. to make the fleas active. Rats are more easily parasitized in this manner as there is some difficulty in holding them for the tube method.

Collection of eggs from parasitized rabbit kittens. Two methods have been used and both have been found to be satisfactory.

(i) Aquarium jars (Fig. 1), eight inches deep with floor space of twelve by seven inches, may be used to house parasitized kittens. Each jar is provided with a close fitting lid covered with forty mesh brass wire gauze. A loose fitting, removable platform of ⅛ inch wire mesh is fitted in the jar and supported on four legs two inches high. A removable tray, consisting of a piano wire frame covered with black organdie, is suspended one inch below the wire mesh platform.

Eggs deposited by the fleas drop through the wire mesh platform and fall on the cloth tray. At each collection of flea eggs the rabbit is taken from the jar and the platform and egg tray are removed. The egg tray is given a sharp tap over a tray to dislodge the flea eggs and foreign material which are deposited on it, the eggs, together with foreign matter, then being tipped into a small vial with the aid of a glass funnel.

(ii) An improved egg collecting cage in which parasitized rabbit kittens can be housed has been devised (Fig. 2). The cage is made of plain galvanized iron and is divided into two compartments by a sliding door. The floor in one compartment consists of ⅛ inch wire mesh and beneath it is a cloth-covered sliding tray on which the eggs are collected. The front of the compartment is provided with a glass window through which the parasitized rabbit can be inspected. The other compartment has a floor of ¼ inch wire mesh beneath which is a plain sliding tray for the collection of rabbit faeces. The cage is placed in a shallow tray containing oil to act as a flea trap.

The collection of eggs has been confined to the period between 9.30 a.m. and 4.30 p.m. The rabbit is placed in the cage at 9 a.m. after being given access to green food from 4.30 p.m. on the previous day. The egg trays are rarely fouled with faeces or urine when the rabbit is fed in this manner.

Separation of eggs. Eggs collected from the trays are mixed with flea faeces, and scurf, hair, etc., from the rabbit. The material is sieved through forty mesh wire gauze to remove the coarser matter. It is then placed in a ½″ by 6″ test tube which is held slightly tilted and rotated slowly. By this means the eggs separate from the foreign material, roll along the tube and are passed into a clean receptacle. This method also separates the badly shaped and damaged eggs which do not roll along the tube readily. Eggs may be counted as they roll out of the tube.

Preparation of the medium and breeding the immature stages. Clean river sand is sieved through a forty mesh wire sieve. About ten per cent. by bulk of dried blood, broken into fairly coarse grains, is mixed with it. This medium when placed one-quarter of an inch deep in a Petri dish 3½ inches in diameter is sufficient to rear one hundred larvae. Single larvae provided with this medium will feed and pupate successfully in small test tubes. It is desirable to have larvae of the same age in one dish so that they will pupate about the same time, thus facilitating collection of the cocoons. A slight degree of moisture is necessary for a successful growth of larvae and this is provided by placing in the breeding receptacles small pieces of filter paper which are moistened daily with water. The eggs are placed on top of the medium and the larvae wriggle into it shortly after they hatch. All breeding is carried out in an incubator at 22°C.

Collection of cocoons and of adult fleas. The larvae are allowed to remain undisturbed in the medium for about six days after the cocoons are completed. This is important as it allows successful pupation, and the hardening of the silken material of the cocoons. The cocoons are then sieved out of the medium with a forty mesh wire sieve, and placed in the adult flea trap (Fig. 3).

The fleas after emerging jump from the small tray containing the cocoons (B) and slide down a glass funnel into an 8″ x 1″ tube (E) containing a little sand; the tubes are removed daily, stoppered with a cork and labelled. A slight degree of moisture is maintained in them with small pieces of moistened filter paper. The fleas in these tubes are stored at 22° C. Agitation or vibration of the tray containing mature pupae induces many to emerge quickly. Many remain within the cocoon and have a long pupal period if left undisturbed.

Experiment on the artificial feeding of adult fleas. A piece of skin removed from a rabbit's ear was stretched taut over one end of a ½″ x 6″ glass tube and held in place with a rubber band. The tube was inverted and suspended vertically within a 1″ x 7″ test tube which contained a little sand, the covered end of the inner tube being kept half-an-inch above the sand.

Freshly emerged fleas were placed in the outer tube, and a small amount of citrated blood in the inner tube. The apparatus was maintained at 22° C. Some fleas attached to the skin and fed on the citrated blood. They were kept alive for twenty-two days, during which time some oviposited. All eggs recovered from these artificially fed fleas failed to hatch, but the observations were confined to one experiment. It was found necessary to renew both blood and skin at five-day intervals. This necessitated detachment and transference of the fleas and was responsible for some mortality and undue disturbance, as the fleas had to be mechanically removed each time. The experiment shows, however, that these fleas may be fed artificially at least for a time.

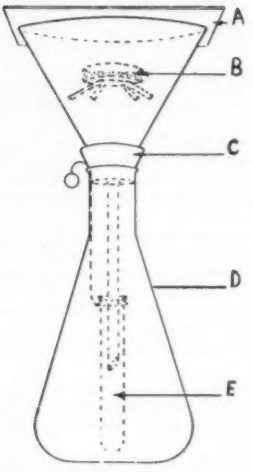

Fig. 3. Adult Flea Trap.

(a) Glass cover over funnel.
(b) Tray for pupa.
(c) Rubber collar.
(d) Glass flask.
(e) Tube for collecting hatched fleas.

Cage for parasitized rabbits. In order to prevent escape of fleas from rabbits used in various experiments the sides of an ordinary rabbit cage were surrounded by a cover made from a single strip of galvanized iron sufficiently long to cover three sides of the cage when bent at two right angles. The ends of the strip were bent at right angles in front of the cage so as to make a slot half an inch wide each side into which a sliding door was fitted. Two strips of galvanized iron two inches wide were soldered to the top edges of the cover, one across the front and the other across the middle, in order to retain the cover in position. A similar strip was soldered across the lower part of the front of the cover and acted as an additional guide for the slide-in door. This covered cage was placed in a galvanized iron tray containing fine sand into which the short legs of the cage and the lower edges of the cover were embedded. The tray was four inches wider and longer than the cage and the sides or walls were eight inches high. This arrangement offered complete protection against the escape of fleas.

SUMMARY.

The life history of *E. myrmecobii* has been described, and details are given of the technique and apparatus used in artificial cultivation of this species in the laboratory.

It was found that the natural food of the larvae was faeces of adult fleas. Dried ox or rabbit blood when mixed in sand provided a suitable artificial medium for the cultivation of larvae.

The optimum temperature for breeding the immature stages was found to be 22° C.

Oviposition has been stimulated during cold weather by raising the room temperature to 22° C.

Special cages have been devised by means of which eggs and faeces of the flea, uncontaminated by the excreta of their host, can be collected.

A trap has been made in which adult fleas may be conveniently collected as they hatch. This is described and illustrated.

Methods used to separate flea eggs from foreign material, and collection of cocoons from the medium, are described.

Rabbits and rats have been parasitized by artificial methods.

A flea-proof cage for housing laboratory rabbits has been devised, and is described.

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REFERENCES.

Parman, D. C. (1923): J. agric. Res. 23, p. 1007.
Sikes, E. K. (1931): Parasitology 23, p. 243.

AN ALKALINE CULTURE MEDIUM FOR DIFFERENTIATION OF STAPHYLOCOCCI AND MICROCOCCI FROM COW'S MILK

by W. I. B. BEVERIDGE

(From the Animal Health Research Laboratory, Parkville, Victoria, Council for Scientific and Industrial Research, Division of Animal Health and Nutrition).

(Submitted for publication 3rd August, 1940.)

During studies on the bacteriology of the bovine udder, the need arose for cultural methods enabling small numbers of haemolytic staphylococci to be detected in milk samples containing large numbers of micrococci.

Preliminary observations. Working with human strains, Chapman *et al.* (1937) devised an alkaline bromthymol blue-agar medium on which most pathogenic staphylococci grew but most non-pathogenic micrococci did not.

We were unable to find any selective effect directly due to bromthymol blue. However, nutrient agar or broth at pH 7·6 gave consistently better growth with haemolytic staphylococci than with non-haemolytic micrococci. Incubation at 43° C., or addition of either potassium permanganate or bromthymol blue accentuated this selective effect, but if the medium were enriched with blood or milk micrococci grew just as abundantly as staphylococci. Knight's synthetic medium (1935) also showed a similar weakly selective effect in a few tests. Staphylococci exposed in broth to a pH of 10·3 survived longer, as shown by subculture, than did micrococci.

Nutrient agar at pH 8·4 supported good growth of haemolytic staphylococci whilst micrococci failed to grow. Addition of blood or milk eliminated this selective effect. When the initial pH of the medium was raised to 9·0–9·2, blood and milk could be added and a clear-cut selective effect obtained; the staphylococcal growth was equal to that on neutral medium, but micrococci grew slightly or not at all in 24 hours. If incubated for 48 hours, micrococci grew in this medium. By further raising the initial pH to 9·4, they could be suppressed for 48 hours or more; this pH, however, prevented haemolysis by some strains of staphylococci.

Procedure. The following is the procedure finally adopted. Tube nutrient agar (pH 7·6) in measured 12 ml. amounts. After autoclaving, determine accurately for each batch of medium the amount of N/2 NaOH required to bring the pH to 9·0–9·2. A satisfactory method is to prepare, aseptically, standard tubes of the medium adjusted to pH 9·0–9·2 (determined electrically) containing 1 ml. of 1 p.c. solution of phenolphthalein and 10 ml. distilled water. To several tubes of each new batch of medium add varying amounts of alkali (usually about 0·3 ml.) together with 1 ml. of phenolphthalein solution and 10 ml. distilled water; cool and compare with standard tubes.

Before sowing, melt the agar in the tubes and add the pre-determined amount of alkali to bring pH to 9·0–9·2. Add 0·6 ml. of blood and mix by rotation. Before adding the inoculum (usually 0·5 ml. of whole or diluted milk) agitate it vigorously by drawing it rapidly in and out of a pipette about 20 times, with the end of the pipette held on the bottom of a sterile test tube. The purpose of this is to break up clumps of cocci. Pour plates and incubate at 37° C. Examine after about 18 hours, and if necessary at 24 hours. The blood and milk bring the pH down by 0·2. If the final pH is over 9·0, haemolysis is suppressed with many strains of staphylococci, and even between pH 8·8 and 9·0 occasional strains show weak or no haemolysis within 24 hours. Therefore, if non-haemolytic colonies of fair size are present at 24 hours, pick some off and sow them on to ordinary blood agar. Bovine blood was used throughout this work.

Results.

By this method it is possible to detect as few as 1 or 2 staphylococci in 0·5 ml. of milk containing large numbers of micrococci.

If the final pH is not above 9·0, most strains of staphylococci grow just as well in alkaline blood-agar and give as many and as large, or larger, colonies as at pH 7·6: moreover they usually show haemolysis. Micrococci usually do not grow in blood-agar at pH 8·8 to 9·0 in 24 hours, but a few minute colonies may grow as satellites around the staphylococcal colonies. Micrococci will grow if large numbers of staphylococci are present or if the plates are incubated longer than 24 hours, or, sometimes, if the milk has not been agitated to break up clumps.

Bovine strains. Twenty-three bovine strains of haemolytic staphylococci from milk were grown on blood-agar at pH 8·8. All except one grew well. All fermented mannite readily.

Of 39 strains of non-haemolytic micrococci isolated from cows' milk, 38 gave no growth or only a trace of growth in 24 hours; one grew rather feebly. Only one strain fermented mannite.

Human strains. Of 40 human strains of cocci recently isolated from apparently normal skin, 7 grew on alkaline blood-agar. One of the latter was a typical *Staph. aureus* (haemolytic, golden colour, coagulase-positive, mannite-positive in one day). Four others produced a deep fawn pigment but were non-haemolytic; the remaining two were non-haemolytic white strains. Of the 33 strains growing not at all or only slightly on alkaline blood-agar, all were non-haemolytic except one which was haemolytic but did not ferment mannite or form coagulase. Mannite-fermentation gave irregular results (negative with 22 strains and positive with 16, which, however, only produced acid after two or more days).

Of 40 human strains of staphylococci isolated from pathological material by workers at the Commonwealth Serum Laboratories and the Melbourne Hospital, and regarded by them as pathogenic *Staph. aureus*, all grew well on alkaline blood-agar. Most had been isolated from one to four years previously, and when tested on neutral blood-agar, eight were non-haemolytic, 24 gave a mixture comprised mainly of non-haemolytic colonies, and eight were haemolytic. All except one fermented mannite in 24 hours.

Tests were also carried out on 22 strains isolated from one to seven years previously by the Commonwealth Serum Laboratories from a variety of conditions including minor skin lesions such as pustules. These were regarded as *Staph. albus* when isolated. Six grew well on alkaline blood-agar (these all fermented mannite during one or two days' incubation), nine grew moderately well (eight negative with mannite, one positive), and seven grew not at all or only slightly (all negative with mannite).

SUMMARY.

Alkaline blood-agar, at a final pH between 8·8 and 9·0, can be used as a selective medium for staphylococci in cows' milk.

Small numbers of staphylococci may be isolated from milk containing large numbers of micrococci by the use of this medium.

Growth on alkaline blood-agar showed a close positive correlation with haemolysis in a series of strains of staphylococci and of micrococci isolated from milk.

The same clear-cut results were not obtained with human strains, but the results suggest that growth on alkaline medium may be a useful indication of pathogenicity in strains of human origin.

Acknowledgments. This study was suggested by Dr. L. B. Bull, and was carried out in the Animal Health Laboratory, Parkville, as part of the investigation on the bacteriology of the udder of the cow being carried out in that laboratory. I am greatly indebted to Dr. F. G. Morgan, to Dr. F. M. Burnet, and to Mr. E. Munch-Petersen for supplying strains of cocci.

REFERENCES.

Chapman, G. H., Lieb, C. W., Berens, C. and Curcio, Lillian (1937): J. Bact., 33, p. 533.
Knight, B. C. J. (1935): Brit. J. exp. Path., 16, p. 315.

STUDIES IN RESPIRATION

3. THE EFFECT OF EXPIRATORY SPEED ON THE DEPTH OF THE OXYGEN TROUGH OF PROGRESSIVE EXPIRATION.

by F. S. COTTON[1]

(From the Department of Physiology, University of Sydney).

(Submitted for publication 29th June, 1940.)

In the following experiments conditions have been standardized in regard to a preliminary rest period, a comfortable sitting posture, a blank rehearsal in the case of each fresh subject, and a standard apnoea period of one minute, except where the rehearsal experiment indicates the necessity for reducing the time in accordance with the capacity of the subject.

An attempt has been made to determine whether any regular relation exists between the rate of expiration and the depth of the oxygen trough, in order to apply the result to the standardization of future experiments.

In most of the earlier experiments (Cotton, 1939a) an attempt was made to empty the lungs as completely as possible. In a number of these experiments it was found that the terminal sample, taken from the tube some three inches from the mouth, showed a slight fall in the oxygen percentage and a slight rise in carbon dioxide. Since the depth of the oxygen trough is the difference between the lowest value for the series of samples and that of the highest terminal sample, this final reduced value was of no special interest. It is therefore more convenient to conclude the expiration without excessive effort, and to use a rather longer tube connecting the last sampling junction with the mouth. Under these circumstances at any rate with the main subject, the final oxygen value was almost invariably the highest.

When it was realized that the depth of the oxygen trough was enhanced by a preliminary state of apnoea, it was thought that undue delay in expiration might diminish the final rise in the oxygen percentage, since that portion of alveolar air, destined to be last expired and richest in oxygen, is probably continuing to lose oxygen throughout the expiration. In any case it is scarcely conceivable that it could be gaining oxygen. On this account the expiration into the long tube system was made reasonably fast. Some slower expirations were made subsequently and, as these yielded deeper oxygen troughs, it was decided to carry out a series of such experiments to contrast the effects of slow and rapid expiration.

TABLE 1.

Slow Expiration.

Expt.	O_2 Samples numbered from the mouth.						Depth of Oxygen	Expt.	CO_2 Samples numbered from the mouth.						Terminal drop of
No.	S_1	S_2	S_5	S_6	S_{15}	S_{17}	Trough.	No.	S_1	S_2	S_5	S_6	S_{15}	S_{17}	CO_2
I.	12·53	12·20	11·13	11·10	11·53	11·67	1·43	I.	6·43	6·52	6·71	6·71	6·51	6·45	0·28
II.	12·69	12·35	11·58	11·64	11·81	11·93	1·11	II.	6·34	6·45	6·57	6·54	6·43	6·32	0·25
III.	12·90	12·76	11·83	11·78	12·19	12·05	1·12	III.	6·43	6·53	6·73	6·78	6·59	6·65	0·35
IV.	12·04	11·89	11·11	11·15	11·37	11·27	0·93	IV.	6·73	6·80	6·95	6·94	6·84	6·84	0·22
Mean	12·54	12·29	11·41	11·41	11·72	11·73	1·15	Mean	6·48	6·57	6·74	6·74	6·59	6·56	0·27

Mean Depth of Oxygen Trough=1·13% Mean Terminal drop of CO_2=0·26%

Quick Expiration.

Expt.	O_2 Samples numbered from the mouth.						Depth of Oxygen	Expt.	CO_2 Samples numbered from the mouth.						Terminal drop of
No.	S_1	S_2	S_5	S_6	S_{15}	S_{17}	Trough.	No.	S_1	S_2	S_5	S_6	S_{15}	S_{17}	CO_2
I.	11·86	11·60	11·55	11·40	11·63	11·57	0·46	I.	6·55	6·62	6·51	6·64	6·56	6·61	0·09
II.	12·44	12·36	12·34	12·44	12·26	12·30	0·10	II.	6·26	6·26	6·33	6·32	6·34	6·25	0·07
III.	13·25	13·30	12·96	12·94	13·10	12·99	0·36	III.	6·21	6·16	6·29	6·25	6·30	6·29	0·09
IV.	13·04	13·00	12·88	12·89	12·92	12·94	0·16	IV.	6·12	6·21	6·24	6·28	6·27	6·20	0·16
Mean	12·65	12·56	12·43	12·42	12·48	12·44	0·27	Mean	6·28	6·31	6·34	6·37	6·37	6·34	0·10

Mean Depth of Oxygen Trough=0·23% Mean Terminal drop of CO_2=0·09%

[1] This work was carried out during the tenure of a senior Research Fellowship under the National Health and Medical Research Council of Australia.

Since it was considered possible that variations in pulmonary conditions on different occasions might influence the depth of the oxygen trough, it was arranged to pair the experiments so as to obtain on each occasion the result of one fast and one slow expiration.

Moreover, in case any given experiment should be influenced by its predecessor, the priority of the "fast" and "slow" expiration experiments was alternated on successive occasions. The series consisted of four such pairs of experiments.

The results of these experiments are set out in Table 1 and Figs. 1 and 2.

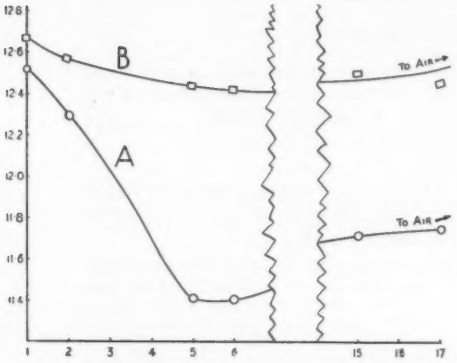

Fig. 1. Comparison of the mean terminal oxygen gradient of expired air (after apnœa). (Same subject for all experiments.)

A. When the rate of expiration was slow.
B. When the rate of expiration was fast.
Abscissa: Samples numbered from the mouth.
Ordinate: Percentage of O_2.

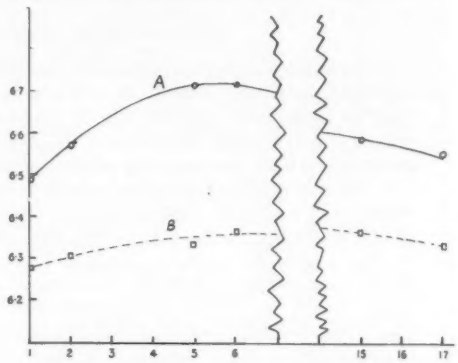

Fig. 2. Comparison of the mean terminal CO_2 gradient of expired air (after apnœa). (Same subject each time.)

A. When the rate of expiration was slow.
B. When the rate of expiration was fast.
Abscissa: Samples numbered from the mouth.
Ordinate: Percentage of carbon dioxide.

In each of the present experiments, nine samples were taken but not all were analysed each time, since it has been shown previously (Cotton, 1939b) that an essentially accurate graph of the resulting values could be plotted by the fixing of a limited number of the points.

In Table 1 are shown the results of the four separate experiments where expiration was fast, and the corresponding four where it was slow. These results are averaged in the bottom row in each group.

The main result stands out quite unmistakably from the data, namely that the mean depth of the oxygen trough in the case of the slow expirations is much greater than that for the fast expirations. The former amounts to an absolute value of 1·13 p.c. of oxygen, and the latter to 0·23 p.c. of oxygen.

The complementary phenomenon in regard to CO_2 is also evident from the mean results, the extent of the terminal drop in CO_2 percentage amounting to 0·26 p.c. for the slow series and to only 0·09 p.c. for the fast series. In the respective final columns of Table 1 are shown the individual values for depth of oxygen trough and for terminal decrease of CO_2.

In the foregoing experiments it was found that the narrow bore of this series of tubes (about 9·5 mm.) imposed a distinct limit upon the maximum speed of expiration. In order to avoid this limitation a series of tubes of larger diameter (approx. 16 mm.) was used. This permitted faster expiration with less effort. The previous series of 19 units, each of volume about 130 ml., permitted the examination of the last 2½ litres of expired air. The new series of units consisted of nine tubes each of about 310 ml. capacity, permitting the examination of the last 2·8 litres of the expired air. Since the terminal tube was connected by a long rubber tube to the recording spirometer, even the far sample could be taken without risk of contamination.

A description of the procedure in such a pair of experiments runs as follows:

1. The subject rested (sitting comfortably) for twenty minutes following a period of average laboratory activity. 2. Pulse and respiration rates[2] were taken during this period.

[2] The pulse and respiration rates were taken with a view to correlation with the depth of the oxygen trough, since it seems quite possible that the latter may vary with the distribution of blood in the lungs or with a variation in rate of blood flow through the lungs. Subsequent examination of the data, however, did not reveal any significant relation of the oxygen trough to either pulse or respiratory rate.

3. A deep breath was taken and held for 60 seconds. 4. This was expired quickly through the tubes, and then the mouth end of the tube system was blocked. This was a preliminary procedure to replace the air in the tubes by expired air under the conditions of the true experiment. The spirometer was then returned to its zero level by temporarily detaching its connection to the tube system. 5. The subject rested for another six minutes. 6. Procedures 3 and 4 above were repeated, a graphic record of the spirometer rise being taken. 7. All nine samples were then taken proceeding in order from 9 to 1, with the mouth end of the system closed. 8. The subject rested for six minutes while fresh sampling tubes were attached at the junctions of the tube units. 9. Procedures 3 and 4 were repeated with slow expiration. 10. Samples were taken as before. The results of the later experiments are set out in Table 2.

TABLE 2.

Expt.	Depth of Oxygen Trough.		Terminal Drop of Carbon Dioxide.	
	Slow Expiration.	Fast Expiration.	Slow Expiration.	Fast Expiration.
1.	0·85	0·51	0·16	0·11
2.	0·88	0·04	0·17	0·08
3.	0·49	0·02	0·20	0·09
4.	0·78	0·22	0·28	0·09
Mean	0·75	0·20	0·20	0·10

The results shown in Table 2 confirm the findings listed in Table 1, the corresponding values in all cases being reduced in accordance with the increased rate of expiration. The slow series of Table 2 was actually faster than the slow series of Table 1, so that the lower value in the later experiments agrees with the general findings.

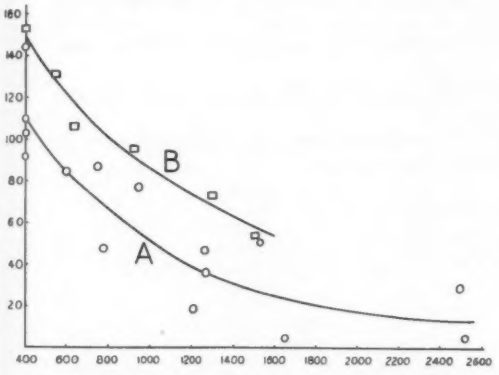

Fig. 3. Showing the effect of rate of expiration on the extent of the oxygen trough (after apnœa).

A. The graph for the main subject of the investigation

B. The graph for a second subject.

Abscissa: Rate of expiration in ml. per second.

Ordinate: Extent of O_2 trough in units of (0·01 p.c.).

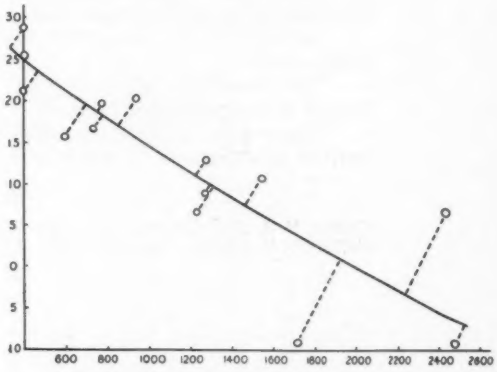

Fig. 4. Showing the effect of rate of expiration on the extent of the terminal drop of CO_2 (after apnœa).

Abscissa: Rate of expiration in ml. per second.

Ordinate: Terminal drop in CO_2 in units of (0·01 p.c.).

Data from the graphic records. Rates of expiration were calculated from the records, and found to vary from 350 ml. per second to 940 ml. per second in the slow series, and from 1,570 ml. per second to 2,500 ml. per second in the fast series.

Hence the data were sufficiently spaced to construct a graph relating the depth of the oxygen trough to the rate of expiration. This graph is shown by the line A of Fig. 3. Similarly the relation between the terminal carbon dioxide drop and rate of expiration is shown in Fig. 4.

Observations on other subjects. To exclude the possibility that these effects represent some individual characteristic, a similar series of experiments was conducted on a second subject who exhibits the phenomenon of the oxygen trough to a moderate degree. The results for oxygen in this case are shown in Fig. 3, line B. The results for carbon dioxide were not sufficiently numerous to draw a representative graph, but they were in general agreement with the data of Fig. 4. It will be seen that the two curves of Fig. 3 are remarkably similar.

Additional experiments were conducted on two more subjects. In one case the depth of the oxygen trough was similarly influenced by rate of expiration, and to a more marked degree as the rate of expiration was reduced. The fourth subject gave small though similar results. Hence the parallelism noted between the results obtained with the first two subjects cannot be regarded as being of a quantitatively general nature.

As far as the slowing of expiration extends in the graphed results there is a continuous tendency to increase the depth of the oxygen trough. This, however, does not imply the absence of some optimal rate for a maximal trough. Some of the increase in the oxygen trough may be due to the extension of the total apnoea period. Further work is necessary to evaluate this factor.

DISCUSSION.

From the results it appears that once certain differences in the composition of the alveolar air have been set up within the lungs, the maintenance of such differences may be considerably influenced by the speed at which the air is expired. Since the primary cause of the phenomenon is unknown, no explanation of this fact can be given.

SUMMARY.

Experiments have been conducted to show that the depth of the oxygen trough of expiration may be greatly influenced by the speed at which the expiration is made.

In all four subjects examined the oxygen trough showed an increase with decrease in the rate of expiration.

In three subjects this effect was considerable, while in the fourth it was relatively small.

The possibility of an optimal rate of expiration for a maximum oxygen trough is not precluded by these experiments.

The results indicate the necessity for standardization of expiratory rate in further investigation of the oxygen trough phenomenon.

REFERENCES.

Cotton, F. S. (1939a): Austral. J. exp. Biol., 17, p. 425.
Cotton, F. S. (1939b): Austral. J. exp. Biol., 17, p. 433.

THE USE OF SLIDE AGGLUTINATION TO DETERMINE PATHOGENICITY OF STAPHYLOCOCCI

by R. CHRISTIE

(From the Commonwealth Serum Laboratories, Parkville, N.2, Victoria.

(Submitted for publication 4th September, 1940.)

The coagulase test is generally regarded as the best single test of pathogenicity in staphylococci (Cruickshank, 1937; Chapman, Berens, Nilson and Curcio, 1938; Fairbrother, 1940).

It was found that unabsorbed sera, prepared in this laboratory from coagulase-positive strains, agglutinated most heterologous coagulase-positive strains but failed to agglutinate coagulase-negative strains (Christie and Keogh, 1940).

It seemed possible, therefore, that agglutination tests with pooled sera prepared with coagulase-positive strains might give results corresponding to those of the coagulase test, and serve as tests for pathogenicity.

An attempt was made to prepare a mixture of sera suitable for this purpose.

Materials and Methods.

Strains used. 392 strains of staphylococci were examined; 335 were of human origin and 57 of animal origin. The sources of these organisms and the results of the coagulase-test are shown in Table 1.

TABLE 1.

Origin of the strains and results of the coagulase tests.

	Source.	Coagulase-positive.	Coagulase-negative.	Total.
Human	Boils	45	—	45
	Osteomyelitis	37	—	37
	Skin infections (acne, etc.)	24	19	43
	C.S.F.	—	2	2
	Pleural fluid	1	—	1
	Lung (P.M.)	1	1	2
	Sputum	10	2	12
	Throat swabs	30	1	31
	Blood culture	15	1	16
	Nasal swabs	26	12	38
	Urine	2	17	19
	Urethral swabs	2	17	19
	Rectal swabs	—	3	3
	Post-nasal sinuses	7	22	29
	Tooth abscesses	3	1	4
	Eye	2	7	9
	Ear	2	—	2
	Unknown and uncertain	19	4	23
	Total—Human	226	109	335
Bovine	Milk (mastitis)	29	6	35
Equine	Pus (sinus)	3	3	6
Feline	Nasal swabs (? distemper)	0	1	1
Canine	„ „ (distemper)	6	4	10
Ovine	Udder	5	0	5
	Total—Animal	43	14	57
	Total—Human and Animal	269	123	392

Preparation of anti-sera. Anti-sera were prepared in rabbits by the method of Cowan (1939), using organisms killed at 60° C. After four weeks' treatment, sera were obtained with a final titre of 1: 200 to 1: 400. They were preserved with merthiolate (1: 10,000) and showed no fall in titre after one year's storage at 4° C.

Preparation of antigen. The strain was grown for 18–24 hours at 37° C. in 0·05 p.c. glucose broth (Lyons, 1937). The culture was then heated to 100° C., centrifuged, and the

organisms re-suspended in physiological saline to give a suspension of 4,000 million organisms per ml.

Where organisms grown on nutrient agar slopes were used, suspension of similar strength was made in saline.

Technique of agglutination test. Using a capillary pipette a small drop of bacterial suspension was placed on a slide, and a drop of diluted serum added with a 28-gauge platinum loop having a diameter of 1 mm. The drop of suspension was found by weighing to be twenty times the volume of the serum drop. A drop of suspension, without serum, served as control. The slide was then rocked for 60 seconds. Agglutination, when it occurred, was usually complete, or nearly so, in 30 seconds.

Technique of the coagulase test. Rabbit plasma was generally used, but when more convenient, human or horse plasma. At times all three were used. The blood was withdrawn and added to an equal volume of 4 p.c. sodium citrate; the cells were removed by centrifuging. A loopful of organisms from a 24-hours' agar slope culture was suspended in 0·5 ml. of plasma. Known positive and negative strains were always included as controls. Readings were made after 3, 5 and 24 hours' incubation at 37° C.

Results.

(a) *Coagulase tests.* 269 strains coagulated plasma, in most cases within three hours; 123 strains did not coagulate plasma. Two animal strains, both $\alpha\beta$ toxin-producers, gave anomalous results. One strain, isolated from the nasal discharge of a dog with distemper, did not coagulate human plasma but coagulated horse and rabbit plasma; the other strain, isolated from a sinus of a horse's withers, did not coagulate horse plasma but coagulated human and rabbit plasma. No similar irregularities were detected amongst the human strains.

All definitely haemolytic strains coagulated plasma. One strain, S5, isolated from the pus of osteomyelitis and which coagulated plasma, showed little or no haemolysis on sheep blood-agar and produced no demonstrable haemolysin when cultivated under increased CO_2 tension in "toxin" broth (nutrient broth with 0·1 p.c. agar). 1 ml. of a 24 hours' broth culture injected intravenously into a rabbit killed the animal in nine days. The organisms recovered from the heart blood and abscesses of the kidney, were coagulase-positive and agglutinable with several anti-sera to the same degree as the original organisms, but they were still non-haemolytic on sheep blood-agar.

(b) *Agglutination tests with organisms grown in broth.* An unabsorbed serum (prepared from a type I strain received from Dr. S. T. Cowan), diluted 1: 10, agglutinated most coagulase-positive strains. Some strains not agglutinated by this serum were agglutinated by a type II serum, while the remainder, with one exception, were agglutinated by a serum prepared from strain S174.

One volume of each of these three sera was pooled and added to seven volumes of saline, so that each serum in the mixture was diluted 1: 10. All strains were tested with this pooled diluted serum; the results are shown in Table 2. Of 269 coagulase-positive strains, 257 were agglutinated, 16 of them incompletely; nine strains were auto-agglutinable. One human strain (S291) and two animal strains were not agglutinated.

Of the 123 coagulase-negative strains, 120 were not agglutinated, one was slightly agglutinated: two were auto-agglutinable.

TABLE 2.

Results of slide agglutination tests with pooled sera and staphylococci grown in broth.

	Coagulase-positive Strains.		Coagulase-negative Strains.	
	Human.	Animal.	Human.	Animal.
Agglutinated	220	37	1	0
Not agglutinated	1	2	106	14
Auto-agglutinated	5	4	2	0
Total	226	43	109	14

The human coagulase-positive organism S291 which was not agglutinated, was further examined. When grown for 24 hours on sheep blood-agar it was weakly haemolytic. In "toxin" broth under increased CO_2 tension it produced a lysin for rabbit cells which was neutralized by α anti-toxin; no β toxin was detected. It coagulated horse, human and rabbit plasma and fermented mannitol. 1 ml. of a 24 hours' broth culture given intravenously killed a rabbit within 24 hours, and an almost confluent growth of the organisms was obtained on blood-agar inoculated with a drop of the heart blood taken *post mortem*. An attempt was made to prepare anti-sera with it but, at the end of the usual course of inoculations in rabbits, the sera agglutinated the organisms only in low dilution. Efforts to raise the titre of the sera by giving formalinized and, finally, live organisms were only partly successful. On testing the sera against other pathogens, they were found to agglutinate some of them in moderately

high titre (1: 100). Strain S291 was apparently related serologically to the pathogens but was of exceptionally low agglutinability.

The two coagulase-positive animal strains not agglutinated were isolated from dogs. Both produced α and β toxins, and when grown in broth and re-suspended in saline formed coarse suspensions as do many coagulase-negative strains. Cowan (1938) found a serological relationship between certain haemolytic animal strains and saprophytic staphylococci.

(c) *Agglutination tests with organisms grown on nutrient agar.* Organisms grown on nutrient agar were less agglutinable than those grown in broth. A serum made from a different strain (S39) from the three used above, was substituted for the type I serum in the pooled serum mixture, and the size of the drop of suspension was reduced to ten times that of the drop of diluted serum. Most coagulase-positive strains were agglutinated by these pooled diluted sera (S39, type II and S174). Three of the first 100 coagulase-negative strains were partly agglutinated. These strains were non-haemolytic on sheep blood-agar, did not produce toxin in broth, did not ferment mannitol, did not coagulate horse, human and rabbit plasma and did not kill rabbits and mice when injected intravenously. Absorption of serum S39 with one of these coagulase-negative strains removed the agglutinins for all three, leaving the agglutinins for the coagulase-positive strains almost unaffected.

Type II and S174 sera were added to this S39 absorbed serum to provide a mixture which would agglutinate all coagulase-positive strains, but partial agglutination of some coagulase-negative strains again occurred. It was found necessary to use the absorbed serum S39 (diluted 1: 5) alone and re-test strains not agglutinated by this serum with a mixture of two sera. A type III serum was found more suitable than serum S174 (see Section (b)), although both sera were equally effective when tested against organisms grown in broth.

As shown in Table 3, the absorbed serum S39 agglutinated 203 (12 incompletely) of 226 coagulase-positive human strains. Using a mixture of type II and S120 sera in which each serum was diluted 1: 10, 16 of the remainder were agglutinated; two strains were auto-agglutinable. When grown in broth two of the remaining five were agglutinated by serum S174, and two by type I serum; the other was the apparently inagglutinable strain S291. No coagulase-negative strain was agglutinated.

Of the 43 coagulase-positive animal strains, 38 were agglutinated by serum S39, three by the type II–S120 serum mixture, and two were not agglutinated. No coagulase-negative strain was agglutinated.

TABLE 3.

Results of slide-agglutination tests with staphylococci grown on nutrient agar. Organisms were first tested with absorbed serum S39; if not agglutinated, they were tested with a diluted mixture of Type II and Type III sera.

	Coagulase-positive Strains.		Coagulase-negative Strains.	
	Human.	Animal.	Human.	Animal.
Agglutinated	219	41	0	0
Not agglutinated	5	2	109	14
Auto-agglutinated	2	0	0	0
Total	226	43	109	14

203 of the human strains and 38 of the animal strains were agglutinated by the absorbed serum S39.

The method can be used to test organisms from single colonies. Sufficient material can usually be collected from a colony with a platinum loop to give an adequate suspension in each of two drops of saline, to one of which the serum is then added.

(d) *Agglutination of coagulase-negative strains.* Attempts to prepare antisera with coagulase-negative strains were not very successful. Five strains giving different biochemical reactions were injected into rabbits. Even after a prolonged course of injections and, finally, large doses of living organisms, two of the sera could not be raised to a workable titre. The other three were tested against 12 selected coagulase-positive strains and 12 coagulase-negative strains. Nearly all the coagulase-positive strains were agglutinated, some to a slightly higher degree than the homologous strains. Eight of the heterologous coagulase-negative strains were not agglutinated.

DISCUSSION.

These results indicate that agglutination with pooled serum and with organisms grown in broth may be used as a laboratory test for pathogenicity. The value of this test is limited by the occurrence of auto-agglutinable strains.

The results also have some theoretical interest in connection with the validity of the coagulase test as a criterion of pathogenicity. Agglutination tests with sera

prepared from pathogenic strains divided the staphylococci into two groups. With one exception (S291), the first group included all strains which were pathogenic as judged by their source (45 from boils and 37 from osteomyelitis), together with all strains (258), which were definitely toxigenic. The one characteristic common to all members of this group was ability to coagulate plasma.

The one exception amongst the human strains (S291), although not agglutinated by the pooled sera made with coagulase-positive strains, was found to be serologically related to the coagulase-positive group.

The second group included no strain which could be judged as pathogenic according to its source and (with the exception of strain S291) no definitely toxigenic strains. Strains of this group did not coagulate plasma.

The result of the pathogenicity test in the rabbit with strain S5 suggests that ability to coagulate plasma or to be agglutinated by a serum prepared with a pathogenic strain may be of greater importance in judging pathogenicity than ability to produce haemolytic toxin in detectable quantity.

The agglutination of most coagulase-positive animal strains by sera made from human pathogenic strains accords with the results of Cowan (1938). Using a precipitin technique he showed a group serological relationship between toxigenic human and most toxigenic animal strains.

The anomalous coagulase reactions (Section (a)) given by the two animal strains confirm Chapman's (1938) conclusion that choice of plasma may affect the result of the coagulase test with strains of animal origin.

Since type I, type II and S174 sera contain together agglutinating antibodies for all pathogenic strains, it seems desirable to include the corresponding strains in polyvalent vaccines.

SUMMARY.

335 strains of staphylococci of human origin and 57 strains of animal origin have been examined.

Of 269 coagulase-positive strains, 257 were agglutinated by a mixture of three antisera prepared from coagulase-positive strains; nine strains were auto-agglutinable and could not be tested. One human and two animal strains were not agglutinated; the human strain was serologically related to the coagulase-positive strains in spite of its inagglutinability with the serum mixture. Of 123 coagulase-negative strains, 120 were not agglutinated, two were auto-agglutinable and one was agglutinated slightly.

An attempt to produce agglutinating sera with coagulase-negative strains which would agglutinate coagulase-negative and not coagulase-positive strains was unsuccessful.

REFERENCES.

Chapman, G. H., Berens, C., Nilson, E. L. and Curcio, L. G. (1938): J. Bact., 35, p. 311.
Christie, R. and Keogh, E. V. (1940): J. Path. Bact., 51, p. 189.
Cowan, S. T. (1938): J. Path. Bact., 46, p. 31.
Cowan, S. T. (1939): J. Path. Bact., 48, p. 169.
Cruickshank, R. (1937): J. Path. Bact., 45, p. 295.
Fairbrother, R. W. (1940): J. Path. Bact., 50, p. 83.
Lyons, C. (1937): Brit. J. exp. Path., 18, p. 411.

METHODS FOR THE PREPARATION OF PURE TOBACCO MOSAIC VIRUS NUCLEOPROTEIN (*MARMOR TABACI* VAR. *VULGARE*, HOLMES)[1]

by RUPERT J. BEST

(From the Waite Agricultural Research Institute, University of Adelaide, South Australia).

(Submitted for publication 1st August, 1940.)

Various methods for preparing purified samples of tobacco mosaic virus have been described (Stanley, 1935, 1936; Best, 1936; Bawden and Pirie, 1937). Some require apparatus not available in the average laboratory or are unnecessarily tedious. In previous publications the author has only outlined the general principles by which his preparations were made. Owing to increasing interest a detailed account is given here.

Time of Harvesting of Plants.

It is a common experience that different harvests vary greatly in the amount of work required to get a pure product. The principal factor responsible for this variation is the light intensity prevailing during the half-day immediately preceding the harvest of the leaves. The best preparations were obtained from leaves which had been removed after a period of darkness. Apparently substances which accumulate in leaves during active photosynthesis render the purification process more difficult. In consequence, all harvests were begun before sunrise or, where this was impracticable, the plants were removed to a dark-room on the day before they were harvested.

The age of the plant at the time of harvesting is another factor. Under glasshouse conditions at Adelaide during the spring, summer and autumn good results are obtained when the plants are inoculated at about the ten-leaf stage of development, and harvested from four to six weeks after inoculation or just before they reach the flowering stage.

It is often an advantage to treat the juice from the older mature leaves separately from that of the younger leaves during the early stages of the purification process.

Treatment of Juice.

The juice is extracted from infected leaves by crushing them in a mortar and pestle and expressing through muslin, or by means of a domestic fruit juice extractor with a monel metal sieve with holes at the feed end only.

Aeration of the juice helps in the production of a good preparation. Immediately after extraction, therefore, a stream of air is passed through the juice at room temperature for a period of about 24 hours. The crude juice is then centrifuged in an ordinary laboratory centrifuge generating about 2,000 g. The virus remains in the clear orange-coloured supernatant and may be precipitated from it either by acidifying it to the isoelectric point of the virus or by adding ammonium sulphate.

Heating. In some circumstances aeration may be replaced by heating the juice to a temperature of 65° C. for half-an-hour to precipitate much of the brown pigment, the removal of which is the most difficult part of the purification process.

Preliminary tests had shown that the infectivity of samples of juice held for half an hour at temperatures of 65°, 70° and 75° C. did not differ significantly from that of the control held at room temperature, and 65° C. was chosen as a safe upper limit.

The procedure adopted as a result of these experiments was to place the juice in a water bath or an oven at 5° above the desired temperature and to keep it there for about two hours. When dealing with quantities of 5 to 10 litres of juice at a time this is not a very convenient method and is only to be preferred when its sterilizing effect on microbial flora is desired. Bawden and Pirie (1937) independently developed a similar heat pretreatment which they recommend for general use.

Precipitation of Virus from Juice.

Earlier methods. The author's earliest preparations were obtained by precipitation of the virus from clarified juice at its isoelectric point (pH 3·4) (Best, 1936a). Subsequent purification of the virus was effected by elution with 0·1 M acetic acid and reprecipitation at its isoelectric point. Pure white preparations of the virus were obtained in this way. These

[1] Glasshouse facilities and part of the cost of these investigations are being provided by the Council for Scientific and Industrial Research.

showed a marked satin-like sheen, and when dissolved in buffer at pH 7 to juice concentration (2 mg. per ml.) the solutions were clear and colourless by transmitted light, but showed a faint opalescence by reflected light. Concentrated solutions were a pale straw colour by transmitted light. Later, elution with acetic acid was omitted. Instead, the precipitated virus was dispersed in water and dissolved by adjusting the pH value to 6·5 to 7 with 0·02 M NaOH.

Standard method. A combination of precipitation with ammonium sulphate and precipitation at the isoelectric point was found to give the best results.

The crude juice is aerated for 24 hours and an equal volume of 30 p.c. ammonium sulphate solution added with vigorous shaking. This is left undisturbed till next day, when the virus and other solid matter will have settled. After syphoning off the clear supernatant liquid the green residue is recovered by centrifuging at about 2,000 g. and is then dispersed in distilled water. This brings the virus into solution. The suspension is centrifuged for an hour to throw out extraneous matter, and the virus recovered by reprecipitation with an equal volume of 30 p.c. ammonium sulphate and immediate centrifuging. At this stage the precipitated virus is white with a tinge of grey impurity. In all operations the virus concentration should never exceed 1 p.c. (i.e. about four times its concentration in the original juice); about 5–6 mg. per ml. is preferable. After three or four precipitations with ammonium sulphate further purification is effected by isoelectric point precipitation.

At this stage a 1 p.c. solution of the virus in water has only a very faint straw colour. To this solution an equal volume of 0·10 M acetate buffer[2] of pH 3·3 is added. The final pH value of the mixture will be about 3·4, or the isoelectric point of the virus (Best, 1936a). The precipitated virus is centrifuged for from three-quarters to one hour, the supernatant poured off and the centrifuge tube nearly filled with water (without disturbing the precipitate). After standing for from four hours to overnight to allow the acetic acid to diffuse from the precipitate into the water, the supernatant is poured off and the virus suspended in enough water to give a virus concentration of between 0·5 and 1·0 p.c.. The suspension is stirred to obtain a fine suspension, and the virus dissolved by the *slow* addition of an 0·02 N solution of NaOH. *The rate of addition should not exceed one drop per second, and the suspension should be vigorously stirred by a motor-driven stirrer during this process.* These precautions are necessary to avoid localized regions of pH value above 7·8 where virus would be inactivated (Best and Samuel, 1936; Best, 1936b).

During the neutralizing process several interesting changes occur. At about pH 4·6 the suspension suddenly clears. This is the most viscous stage, and if the concentration is much in excess of 1 p.c. the virus, if undisturbed, will form a fairly rigid gel. Vigorous stirring will restore fluidity to this thixotropic gel. If the viscosity indicates a high virus concentration, additional water should be added to reduce the concentration to about 0·5 to 0·7 p.c. As the pH approaches 6 the viscosity decreases rapidly and at pH 7 the solutions are quite fluid.

The volume of NaOH added at the "clearing point" is a reliable index of the volume required to bring the pH value to 7. Naturally the ratio $\frac{\text{volume NaOH to "clearing point"}}{\text{volume NaOH to pH 7}}$ is dependent on the extent to which excess acetic acid has been removed from the precipitated virus before dispersal. During these early stages in the purification where only the gross excess is removed the ratio is about 1: 3. At the final stages when the virus solution is being prepared as salt free as possible, the ratio is about 1: 6 or higher.

When a pH of between 6·5 and 7 is reached, the solution is centrifuged at about 2,000 g. for one hour. Small amounts of impurities (mainly brown pigment) and white, water-insoluble material, not removed by the ammonium sulphate treatment, are thrown down. This process is repeated until no further purification can be effected. Usually three or four isoelectric precipitations are sufficient, but occasionally many more are necessary. At least three precipitations should always be made in order to remove the ammonium sulphate from the virus.

During the final isoelectric treatment care is taken to remove the excess acetate buffer by diffusion (using several changes of water) before the final solution. This is facilitated by leaving the coarse virus suspension, after one washing with water, in the centrifuge tubes overnight in a refrigerator. This precipitate is then consolidated by centrifuging, and several changes of water added as before to remove residual acetate by diffusion.

Although not essential, the author has found that routine measurements of the electrical conductivity of the "salt free" solutions were very useful in preparing standard solutions of virus.

A polariscope constructed with a pair of polaroid screens is particularly useful for routine examination of preparations during the purification procedure. The concentration of solutions may be very closely estimated by observing the polarization colours of the agitated sols and the period of relaxation of the stream double refraction.

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[2] This buffer is made up by adding 20 ml. of 0·2 M NaOH solution to 50 ml. of 2M acetic acid and then sufficient water to bring the total volume up to one litre.

Comparison of infectivities of virus isolated by various methods. Samples of virus prepared by the methods mentioned were comparable in respect to chemical composition and degree of infectivity per unit weight when tested by the primary lesion method.

Low temperature storage. Solutions of the virus are stored in a refrigerator at about 1° C. Solutions of pure virus prepared in July, 1935, and stored at 1° C. are as active now as when first prepared.

Throughout the purification process solutions of the virus are kept in the refrigerator while not being handled.

Preparation of dried samples of virus. Dried samples for chemical analysis may be obtained from these preparations in many ways. The author has found the following method most suitable.

The virus is precipitated from aqueous solution by the addition of acetate buffer of pH 3·3 and is consolidated by centrifuging. When most of the acetate has been removed as described above the virus is stirred up with absolute alcohol and again consolidated by centrifuging. It is extracted twice more with absolute alcohol and once with dry acetone. When centrifuging the suspension in acetone the tube should be stoppered. After pouring off the acetone the precipitate is transferred to a vacuum desiccator (without desiccant), and the pressure reduced to remove most of the acetone. It is next transferred to a watch glass and placed in an air oven at about 40° C. Finally it is ground in a mortar and bottled in an air-dry condition as a fine, white powder containing from 10 to 12 p.c. moisture. Before use samples of this powder may be dried by heating in an oven at 105° C. for one hour and cooling in a desiccator over P_2O_5.

SUMMARY.

A method is described for preparing pure samples of virus. The removal of pigments and the preparation of a clean white product is greatly facilitated by harvesting the leaves of infected plants immediately after a period of darkness.

After a preliminary aeration of the juice the virus is precipitated by the addition of ammonium sulphate (to 15 p.c.). From this precipitate the virus is extracted with water and insoluble impurities removed by centrifuging. After several repetitions of this process the virus is precipitated by adding an equal volume of acetate buffer solution of pH 3·4. The isoelectrically precipitated virus is collected, redispersed in water and dissolved by the slow addition of 0·02 N sodium hydroxide solution. After centrifuging, the process is repeated.

A method of preparing dried samples of the virus for analysis is described.

REFERENCES.

Bawden, F. C. and Pirie N. W. (1937): Proc. Roy. Soc. (Lond.), 123 (B), p. 274.
Best, Rupert J. (1936a): Austral. J. exp. Biol., 14, p. 1.
Best, Rupert J. (1936b): Austral. J. exp. Biol., 140, p. 323.
Best, Rupert J. and Samuel, Geoffrey (1936): Ann. Appl. Biol., 23, p. 509.
Stanley W. M. (1935): Science, 81, p. 644.
Stanley, W. M. (1936): Phytopathology, 26, p. 303.

CHEMICAL COMPOSITION OF THE BONES OF SHEEP IN WESTERN AUSTRALIA

by E. J. UNDERWOOD[1]

(From the Department of Agriculture, Western Australia).

(Submitted for publication 31st August, 1940.)

During the last six years investigations have been undertaken into the nutritional status of merino sheep, in those parts of the Western Australian wheatbelt where botulism occurs. In the late summer months, sheep in these areas develop a depraved appetite or pica for carrion such as rabbit carcases which may carry the toxin of *Clostridium botulinum* (Bennetts, 1933). The pica is a sarcophagia rather than an osteophagia since the sheep are interested principally in the flesh and viscera of the carcases and rarely, if ever, in the bones alone. The feeding of phosphatic licks to sheep, either young or old, during this period has no effect either on the pica or on their growth or on blood inorganic P levels. It was concluded therefore (Underwood and Shier, 1935, 1936) that phosphorus deficiency could not be a limiting factor in the nutrition of sheep in these areas. Further work has amply borne out this finding for sheep, though there is some evidence (Underwood, 1936) that cattle in the same areas may show signs of phosphorus deficiency. Subsequent experiments (Underwood, Beck and Shier, 1939) have established a lack of available protein and energy for the grazing sheep as the prime factor responsible for the pica and for the live-weight losses in the summer period.

The absence of any sign of phosphorus deficiency was surprising in view of the very low phosphorus content of the dry pastures for several months during the summer. Such pastures consist largely of poor annual species which quickly dry off in the early summer and shed their seed which is largely inedible, leaving dry herbage of exceedingly poor quality (Underwood, Shier and Harvey, 1937). In the growing period (May–October) such pastures contain from 0·18–0·26 p.c. P on the dry matter, but these levels fall to about 0·12 p.c. P in the early summer (December) and to about 0·07 p.c. by March and April. These figures are from samples selected to conform as far as possible to the material actually consumed by the sheep, and suggest either that the sheep's phosphorus requirements are lower than have been previously supposed—a suggestion already put forward by Marston (1934, 1935) and by Underwood and Shier (1936)—or that the other nutritive deficiencies of the grazing, notably available protein and energy, may have the effect of reducing the dietary phosphorus requirements. A further possibility is that they have drawn upon their skeletal reserves. If this were so, bone analyses should be of interest for comparison with values obtained from sheep receiving a good diet containing ample phosphorus and with values from sheep under similar grazing conditions but receiving a phosphorus supplement.

Materials and methods. The death from botulism of eleven 4-tooth (i.e. 2½–3 years) merino ewes from the 1935–6 experiments at Meckering (Underwood, Beck and Shier, 1939) enabled samples of bones to be obtained. Eight of these animals had been on typical dry grazing for a period of 5–6 months prior to death, while three had received the same grazing plus a phosphorus supplement of 0·5–2·5 gm. P daily for 3–4 months before death.

In May, 1940, four merino wethers were killed and samples of the bones obtained. These sheep were only 12–13 months of age, i.e. still growing actively, and for 6–7 months prior to death had subsisted entirely on the dry grazing available at the Merredin Research Station. The late appearance of the 1940 autumn rains at Merredin made these sheep very suitable to the purpose of the investigation since they had had an exceptionally long period on the poor dry grazing.

Immediately after death one femur and one humerus were obtained from each of the

[1] Published with the permission of the Under-Secretary.

sheep. In addition the sixth and seventh rib and the three middle cervical vertebrae were obtained from the Merredin sheep. The bones were cleaned and weighed, placed separately in a linen bag, and roughly crushed before extraction with petroleum ether in a large Soxhlet extractor. The fat-free residue was dried in an oven and ground to a fine powder for analysis. Estimations of total ash, calcium and phosphorus were made on all samples, and nitrogen content was also estimated in some cases.

Results. The fresh weights of the femur and the humerus of all except three of the sheep were obtained. The mean weight of the femurs of six of the mature ewes from Meckering on dry grazing only was 98 gm. and of the humerus 76 gm. The figures for two similar ewes which had received a phosphorus supplement were 99 gm. and 77 gm. respectively. The mean weight of the femur of the four young wethers from Merredin was 106 gm. and of the humerus 89 gm.

The results of the chemical determinations are given as percentages of the oven-dry fat-free bone in Tables 1, 2 and 3. Table 1 contains the results from the eight mature ewes which

TABLE 1.

Composition of Long Bones of Ewes; Dry grazing without P supplement. Percentages on oven-dry fat-free basis.

Sheep No.	Total Ash.	CaO.	P_2O_5.	Nitrogen.	CaO. / P_2O_5.	Ash. / Organic matter.
170	64·1	33·3	27·1	—	1·23	1·79
135	66·1	36·2	25·6	4·75	1·41	1·95
102	62·0	33·6	24·9	4·54	1·35	1·64
107	60·6	32·5	24·3	4·72	1·34	1·54
62	64·4	35·6	26·8	4·67	1·33	1·81
66	65·3	34·8	26·7	4·69	1·30	1·88
156	64·3	34·1	25·7	—	1·32	1·80
61	64·5	34·6	25·8	—	1·34	1·82
Mean	63·9	34·3	25·8	4·67	1·33	1·78

TABLE 2.

Composition of Long Bones of Ewes; Dry grazing plus P supplement. Percentages on oven-dry fat-free basis.

Sheep No.	Total Ash.	CaO.	P_2O_5.	Nitrogen.	CaO. / P_2O_5.	Ash. / Organic matter.
13	64·8	35·1	24·7	—	1·42	1·84
155	65·5	34·7	26·1	—	1·33	1·90
121	62·4	33·5	24·1	4·84	1·39	1·66
Mean	64·2	34·4	25·0	4·84	1·38	1·80

TABLE 3.

Composition of Bones of Wethers; Dry grazing without P supplement. Percentages on oven-dry fat-free basis.

Sheep No.	Total Ash.	CaO.	P_2O_5	CaO / P_2O_5	Ash. / Organic matter.
		Mean of Femur and Humerus.			
1	63·5	33·8	26·2	1·28	1·74
2	63·0	32·2	26·1	1·23	1·70
3	64·4	33·0	26·9	1·23	1·81
4	63·9	32·2	26·5	1·22	1·77
Mean	63·7	32·8	26·4	1·24	1·75
		Sixth and Seventh Ribs.			
1	63·8	31·4	24·8	1·27	1·76
2	62·9	31·6	24·9	1·26	1·69
3	63·8	31·1	24·5	1·27	1·76
4	61·5	31·5	24·8	1·27	1·59
Mean	63·0	31·4	24·7	1·27	1·70
		Middle three Cervical Vertebrae.			
1	49·8	25·9	21·0	1·23	0·99
2	49·7	25·0	20·6	1·21	0·99
3	54·3	27·9	22·7	1·23	1·19
4	53·0	26·8	21·8	1·23	1·13
Mean	51·7	26·4	21·5	1·23	1·07

died from botulism at Meckering. Table 2 contains the analyses of the bones of three similar ewes receiving additional phosphorus. In these two Tables each figure represents the average of duplicate analyses of the femur and the humerus from each sheep. Little or no difference in the composition of these two bones was found. Table 3 contains the results from the same two bones taken from the Merredin wethers, and in addition the mean figures for the composition of the sixth and seventh ribs and of the three middle cervical vertebrae.

DISCUSSION.

No figures for the weight of the bones of merino ewes of this age (2½–3 years) or of 12–13 months old merino wethers appear to be available from elsewhere for purposes of comparison. The similarity of the weights from the ewes with and without additional phosphorus is significant, however, in view of the finding of Martin and Pierce (1934) that a deficiency of phosphorus for a period of twelve months during the growing period produced a considerable lowering of the fresh weight of the bones compared with similar sheep adequately fed. The length, in the case of the long bones, was found by these workers to be unaltered. The heavier weights of the bones of the Merredin sheep, compared with those of the older sheep from Meckering, is of some interest. The climatic conditions at the two places and the apparent grazing conditions are closely similar, but the production of heavier "bigger-boned" sheep at Merredin is a fact well-known by practical men. Whether this is related to the heavier soil types predominant at Merredin compared with those at Meckering is not known, but is under investigation at the present time.

An examination of the chemical data in Tables 1 and 2 discloses that no appreciable differences in the two sets of bones exist. It is evident that the sheep on the dry grazing alone, in spite of its low phosphorus content (0·12–0·07 p.c. P on the dry matter) have not found it necessary to draw noticeably upon the reserves in their long bones.

The figures for composition of the long bones of the younger sheep from Merredin, after an even longer period on similar dry grazing (Table 3), are closely similar and compare very favourably with analyses given by Stewart (1935) for such bones from English breed ewes which had received a good ration containing ample phosphorus for a period ranging from 12–18 months. The mean figures for the femur and humerus of six of such ewes, expressed as percentages on the oven-dry, fat-free bones, are as follows: Ash, 62·4; CaO, 34·7; P_2O_5, 20·7; Ash/organic matter, 1·66. Evidence of the effect of a phosphorus-deficient diet on the mineralization of these bones is given by the following mean figures for seven similar ewes maintained on a low phosphorus, but otherwise adequate, diet for the same periods: Ash, 53·8 p.c.; CaO, 30·4; P_2O_5, 20·7; Ash/organic matter, 1·18. A similar effect on the composition of the long bones was found by Martin and Pierce (1934) with merino sheep maintained for about 12 months on a phosphorus-low diet.

The possibility that the phosphorus intake during the summer period might be low enough to have some effect on the bones of the skull, the ribs of the vertebrae, though not on the humerus or femur, could not be overlooked since both Stewart (1935) and Martin and Pierce (1934) have shown these bones to be more readily drawn upon than the long bones in phosphorus deficiency. The mean composition of the middle three cervical vertebrae and the sixth and seventh ribs of the four Merredin wethers was therefore obtained and is presented in Table 3. The lower mineral content of these bones, particularly the vertebrae, compared with the femur and humerus, is very apparent. This cannot be attributed to the effects of phosphorus deficiency, however, since such bones normally contain much less ash than do the long bones. Thus Stewart (1935) found the mean composition of the ribs of six well-fed English breed ewes to be as follows: Ash, 58·5 p.c.; CaO, 32·5; P_2O_5, 22·5; Ash/organic matter, 1·04. It is evident that the ribs and vertebrae of the Merredin wethers compare very favourably in composition with the "normals" taken from the work of Stewart, and do not show the slightest sign of having been drawn upon as a source of phosphorus.

In comparing the results obtained in the present investigation with those of Stewart and of Martin and Pierce, it should be borne in mind that the sheep examined by these workers were subjected to a much longer period on a phosphorus-low diet. It is possible therefore that if the poor grazing conditions with their low phosphorus levels were maintained for a further few months some effect on the composition of the bones might be brought about. This is not likely to happen in

the agricultural areas in Western Australia, however, and can be ruled out as a practical possibility.

A further difference from other similar investigations is that the Merredin and Meckering sheep have a diet which is low not only in P but also in available protein and energy. Whether such additional deficiencies have the effect of lowering the P requirements of sheep has yet to be shown experimentally. This is a phase of phosphorus metabolism which badly needs further investigation. In the present circumstances it is not likely to be of great practical significance since it is difficult to imagine any protein or energy supplements fed in practice during the summer months which would not at the same time raise the phosphorus status of the sheep's diet.

SUMMARY.

The weights and chemical composition of the femur and humerus from eleven 4-tooth merino ewes after 5–6 months' grazing on poor quality, dry summer herbage in Western Australia and four weaner merino wethers after 6–7 months on similar poor grazing are presented. The chemical composition of specimens of the ribs and vertebrae from the wethers is also given.

No difference either in the fresh weight or the composition of the bones was found between eight of the ewes receiving the poor grazing only and three receiving the same grazing plus a phosphatic supplement. The chemical composition of these bones and that of the young wethers was closely similar to that of well-fed English sheep.

It is concluded that phosphorus deficiency is not, as indicated in earlier publications, a limiting factor in the nutrition of sheep in the agricultural areas of Western Australia.

The relation of these findings to those in other investigations is discussed.

REFERENCES.

Bennetts, H. W. (1933): J. Commonwealth C.S.I.R., 6, p. 137.
Marston, H. R. (1935): *Ibid.* 8, p. 293.
Marston, H. R. (1934): Commonwealth C.S.I.R. Bull., 85.
Martin, C. J. and Pierce, A. W. (1934): *Ibid.* Bull., 77.
Stewart, J. (1935): Report Inst. An. Path. Camb., 4, p. 179.
Underwood, E. J. (1936): Unpublished data.
Underwood, E. J., Beck, A. B. and Shier, F. L. (1939): Austral. J. exp. Biol., 17, p. 183.
Underwood, E. J. and Shier, F. L. (1936): Austral. J. exp. Biol., 14, p. 77.
Underwood, E. J. and Shier, F. L. (1935): J. Dept. Agric. W.A., 12, p. 277.
Underwood, Shier and Harvey, R. J. (1937): *Ibid.* 14, p. 442.

STUDIES IN THE EPIDEMIOLOGY OF Q FEVER

6. THE SUSCEPTIBILITY OF VARIOUS ANIMALS.

by E. H. DERRICK, D. J. W. SMITH AND H. E. BROWN

(From the Queensland Department of Health and Home Affairs, Brisbane).

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As a first step to define the animal reservoir of Q fever we have tested nine species of bush animals, comprising seven rodents and two marsupials, and have found all to be susceptible.

In an early paper (Derrick, 1937) it was incorrectly stated that rabbits were insusceptible to Q fever. To correct this, notes are added on the results of inoculation of two rabbits.

At least two individuals of each species were inoculated intraperitoneally with liver or spleen emulsion from guinea-pigs infected with the stock (J) strain of Q fever. One was killed after about a week and its blood and liver or spleen tested for infectivity by guinea-pig inoculation. When possible 0·01 gm. of blood or viscus was tested as well as larger amounts. From time to time blood was taken from the other animal by heart puncture for guinea-pig inoculation and serum agglutination tests; this procedure was not always successful.

All febrile reactions in test guinea-pigs were checked, either by passage, including inoculation of Q-immune guinea-pigs, or by reinoculating the test guinea-pig six weeks later with stock Q virus to prove its immunity. If a guinea-pig inoculated from a bush animal did not show a febrile reaction in ten to fourteen days it was killed and its organs inoculated into a second guinea-pig.

Temperature charts of some of the animals are given in Fig. 1, and the results of the guinea-pig inoculations and serum-agglutination tests are set out in Table 1. Cases where the infection of the test guinea-pig was inapparent are distinguished in the table, for they presumably indicate smaller doses of virus than the obvious infections. (The incubation period of Q fever in guinea-pigs is occasionally longer than ten to fourteen days; so that some of the infections called inapparent would have become obvious if the guinea-pig had been observed longer instead of being killed for passage).

Rattus assimilis Gould is a common rat in the forest country of Eastern and Southern Australia. Its distribution includes the known Q fever area and extends far beyond it.

Two females, caught in the bush near Imbil, Queensland, were inoculated. There was no temperature reaction. The first was killed on the seventh day, and its spleen found slightly enlarged. Q virus was recovered from 2 ml. of blood and from 0·01 gm. of liver. From the second rat, of which the blood only was inoculated into guinea-pigs, the virus was not recovered.

Rattus conatus Thomas, the dusky field rat, is the common rat in the sugar-cane fields of North Queensland from Mackay to Mossman, and has attained notoriety as a carrier of leptospirosis. It has not been found in the Q fever area of South Queensland. Till recently it was confused with *R. culmorum*.

Mr. W. A. McDougall, of the Central Sugar Experiment Station, Mackay, kindly sent us a number of these rats born in captivity. Four were inoculated with Q fever, *R. con. 1* and *2* at the age of 4½ months, *R. con. 3* and *4* at 8 months. With two of the animals there was a temperature reaction for one day (Fig. 1).

R. con. 1 was killed on the fourteenth day and the virus recovered from a liver-spleen emulsion but not from the blood. *R. con. 2* was accidentally killed on the third day. From *R. con. 3* the virus was recovered from the blood (third day) and the liver-spleen (fifth day). The spleen was slightly enlarged. *R. con. 4* died after heart puncture on the seventh day. The virus was recovered from blood and liver-spleen.

Rattus culmorum culmorum Thomas and Dollman. Several varieties of *R. culmorum* have been described, each of which appears to have a restricted habitat (Troughton, 1939). The type specimen was described from Inkerman, North Queensland; subspecies are found at Cape York, North Queensland; on Moreton Island, South Queensland (*R.c. youngi*); in the Upper Darling region of New South Wales; and on Eyre's Peninsula, South Australia.

Specimens of *R.c. culmorum*, born in captivity at Mackay, Queensland, were obtained through the courtesy of Mr. McDougall, and two males were inoculated. Neither showed any temperature reaction. From the first rat the virus was recovered from the blood on two occasions, and also from a mixed liver-spleen emulsion when the rat died under chloroform anaesthesia on the sixteenth day. The second rat also died under chloroform—on the fourth day—and the virus was obtained from its liver-spleen.

Rattus culmorum youngi Thomas. The finding of agglutinins for *R. burneti* in the serum of a captured *R.c. youngi* has been reported (Freeman *et al.*, 1940).

Three of these rats, born in the laboratory from parents captured on Moreton Island, were inoculated when nearly six months old. Their temperatures were irregular (Fig. 1). *R.c.y. 20* was killed on the seventh day, and 0·01 gm. of both blood and liver proved infective for guinea-pigs. No abnormalities were noticed in the organs. The spleen was not enlarged. Rickettsiae were not found in a liver smear. *R.C.y. 21* died on the twenty-second day, following heart puncture. The virus was not recovered from it, but agglutinins were demonstrated in the blood. The serum of *R.c.y. 22* showed well marked agglutination.

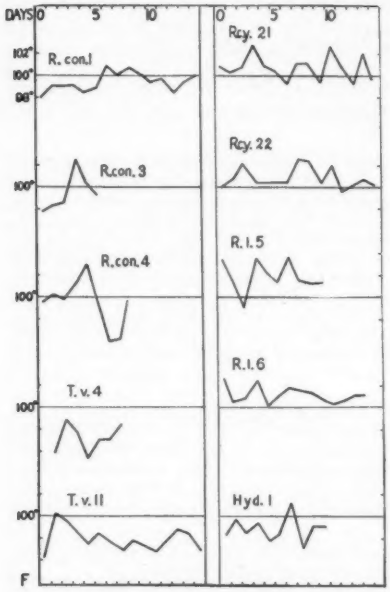

Fig. 1. Temperature charts of some of the inoculated animals. All animals were inoculated on Day 0.

Rattus lutreolus Gray, the eastern swamp-rat, is found in swampy country from South Queensland to Tasmania. The South Queensland form is classed by Troughton as a distinct sub-species *R.l. imbil*.

Two males, captured in the bush near Imbil, Queensland, were inoculated. The temperature of *R.l.5* was irregular, but appeared to have a high normal level (Fig. 1). When the rat was killed on the ninth day its spleen appeared rather large. No rickettsiae were seen in smears of spleen or liver. The virus was recovered from 0·01 gm. of liver, but not from the blood. *R.l.6* showed no temperature reaction. The virus was recovered from its blood on the sixth day, and on the 20th day its serum developed significant agglutinins.

Hydromys chrysogaster Geoffroy. This water rat is fairly plentiful in the streams, swamps and estuaries throughout Eastern Australia. Two captured water rats possessed agglutinins for *R. burneti* in their serum (Freeman *et al*, 1940).

Two water rats were inoculated. *Hyd. 1* was a young rat caught on the bank of the Brisbane River under a wharf in the city. There was a rise of temperature to 101·1° F. on the sixth day, which may have been a Q fever reaction (Fig. 1). The chart is incomplete as the rat escaped on the tenth day and was not recaptured till the fourteenth. No abnormalities were noted in the organs at necropsy on the latter day. The virus was recovered from the liver-spleen. *Hyd. 3* was an adult female captured at Nambour. It died five days after inoculation probably from starvation for it had declined to eat in captivity. The organs appeared normal. The virus was recovered from blood and from liver-kidney-spleen.

Melomys littoralis Lonnberg. This small climbing rat inhabits the coastal districts of Queensland from North to South. A pair, captured by Mr. McDougall at Mackay, were inoculated. One died by accident, and reinoculation of its organs was not possible. The other died on the seventh day from accidental injuries. Virus was recovered from 0·01 gm. of its liver.

Thetomys gracilicaudatus Gould. The Queensland mouse has been recorded from both the North and South of the State. Two females, captured at Mackay by Mr. McDougall, were inoculated. Their temperatures were irregular. *Th.g.1* was killed on the seventh day and the virus recovered from 0·5 ml. of blood and from 0·01 gm. of liver. Rickettsiae were not seen in smears of the liver or spleen. *Th.g.2* was killed after six weeks. The organs were not then infective, but the serum showed significant agglutination.

Trichosurus vulpecula Kerr. This opossum is common in forests all over Australia Two male opossums were inoculated. *T.v.4*, which was captured at Nambour, showed no temperature reaction (Fig. 1). It was killed on the seventh day. Virus was recovered from the spleen. *T.v.11* was captured in a Brisbane suburb. The temperature was raised to 100·2° F. on the day after inoculation and slightly raised the following day (Fig. 1). Agglutinins for *R. burneti* developed in the serum.

Æpyprymnus rufescens Gray. The rufous rat-kangaroo is found throughout Eastern Australia and is common in parts of Queensland.

Two females, caught in the Nambour district, were inoculated. Neither showed a febrile reaction. The first was killed on the eighth day. The spleen appeared swollen. Microscopical examination of the liver showed numerous areas of focal necrosis. Virus was recovered from 3 ml. of blood and 0·01 gm. of spleen. The other rat-kangaroo died on the fifth day, apparently from starvation. It had refused to eat in captivity. There was some congestion of the lungs but no naked-eye or microscopic abnormalities of the liver. The virus was recovered from the blood.

Rabbit. The imported rabbit (*Lepus cuniculus*), run wild, has penetrated into South-West Queensland, but is not present in the known Q fever area.

Two rabbits of the laboratory stock were inoculated. Neither showed any rise of temperature. One was killed on the seventh day. There were no obvious post-mortem abnormalities. Virus was not recovered from the blood but it was obtained from 0·01 gm. of spleen. Blood from the other rabbit was also inoculated without success, but its serum developed agglutinins.

The rabbit is therefore susceptible to Q fever, the infection being inapparent.

TABLE 1.

Results of Guinea-Pig Inoculations and Serum Agglutination Tests.

	Guinea-Pig Inoculation.				Serum Agglutination			
Species and Serial Number.	Day after Infecting Dose.	Inoculum.	Dose: Ml. or Grams.	Result.	Day after Infecting Dose.	1/10	1/30	1/100
R. assimilis 1	7	Blood	2	+ (inap.)				
		Blood	0·01	−				
		Liver emulsion	2·5	+				
		Liver	0·01	+				
R. assimilis 2	7	Blood	1·4	?	7	−		
	14	Blood	0·6	−	14	−		
					21	+	−	
R. conatus 1	14	Blood	3	−				
		Liver-spleen emulsion	1·5	+				
R. conatus 3	3	Blood	1	+				
	5	Liver-spleen emulsion	1·5	+				
R. conatus 4	7	Blood	0·2	+ (inap.)				
		Liver-spleen emulsion	1·5	+				
R.c. culmorum 1	7	Blood	1	+	7	−		
	14	Blood clot	0·8	?	14	−		
	16	Blood	0·5	+ (inap.)				
	16	Liver-spleen emulsion	2	+ (inap.)				
R.c. culmorum 2	4	Liver-spleen emulsion	2	+				
R.c. youngi 20	7	Blood	1	+ (inap.)	7	−		
		Blood	0·01	+ (inap.)				
		Liver emulsion	2	+				
		Liver	0·01	+				
R.c. youngi 21	10	Blood	0·5	−	10	−	−	
	22	Liver-spleen emulsion	2	−	21	++	−	
R.c. youngi 22	13	Blood	1	−	13	Tr.	−	
					21	+	−	
					29	++	−	
					36	+++	++	
					60	+++	+++	−
R. lutreolus 5	9	Blood	1·5	−	9	−		
		Blood	0·01	−				
		Liver emulsion	2	+				
		Liver	0·01	+ (inap.)				
R. lutreolus 6	6	Blood clot	1	+ (inap.)	6	−		
	13	Blood clot	1	−	13	−		
					20	+++	−	
					33	+++	−	
H. chrysogaster 1	14	Liver-spleen emulsion	2	+ (inap.)				

TABLE 1 (continued)

Species and Serial Number.	Guinea-Pig Inoculation. Day after Infecting Dose.	Inoculum.	Dose: Ml. or Grams.	Result.	Serum Day after Infecting Dose.	Agglutination 1/10	1/30	1/100
H. chrysogaster 3	5	Blood clot	1	+				
		Liver-kidney-spleen emulsion	3	+				
M. littoralis 5	7	Liver emulsion	3	? (died)				
		Liver	0·01	+				
Th. gracilicaudatus 1	7	Blood	0·5	+ (inap.)				
		Liver emulsion	4	+				
		Liver	0·01	+ (inap.)				
Th. gracilicaudatus 2	42	Liver emulsion	4	−	42	+++	+	
		Spleen emulsion	2	−				
		Kidney emulsion	2	−				
T. vulpecula 4	7	Blood	3	? (died)	0	−		
		Blood	0·01	−				
		Spleen emulsion	2·2	+				
		Spleen	0·01	−				
T. vulpecula 11	7	Blood	3·5	−	0	−		
	10	Blood clot	0·3	−	7	−		
	14	Blood clot	1·5	−	14	−		
	56	Liver emulsion	3	−	21	++		
	56	Spleen emulsion	3	−	28	++		
	56	Kidney emulsion	3	−	35	+	−	
					42	+++	−	
					49	+++	+++	−
					56	+++	+++	−
AE. rufescens 1	8	Blood	3	+	0	−		
		Blood	0·01	−				
		Liver-spleen emulsion	2	+				
		Spleen	0·01	+				
AE. rufescens 3	5	Blood	3	+ (inap.)	0	−		
		Liver emulsion	1·9	? (died)	5	−		
Rabbit 26	7	Blood	4	−				
		Blood	0·01	−				
		Spleen	0·77	+				
		Spleen	0·01	+ (inap.)				
Rabbit 27	7	Blood	2	−	0	−		
					7	−		
	10	Blood	3	−	10	+++	−	
	14	Blood	3	−	14	+++	−	
					24	+++	−	

inap. means that the infection of the test guinea-pig was inapparent..
Tr. = Trace.

DISCUSSION.

Recovery in a guinea-pig of Q virus from the inoculated animal was accepted as demonstrating its susceptibility. All nine species were more or less susceptible. With five the virus was recovered from at least two inoculated individuals, with the other four from one only. Recovery of virus was less likely when blood only was available for inoculation. Liver or spleen was infective in 14 of 15 animals tested within 16 days of inoculation. (The only failure was due to death of the test guinea-pig from a contaminating organism.) With six animals (representing six species) as little as 0·01 gm. of liver or spleen proved infective, but only once was a similar amount of blood found infective.

With four species significant agglutination for *R. burneti* was demonstrated. In the case of *R.c. youngi 22* and *T. vulpecula 11* a rise in the agglutinin titre oc-

curred as late as five and seven weeks after inoculation. This late rise is good evidence that these two animals, from which virus was not recovered, were infected.

A noteworthy feature was the mildness of the infection throughout the series. Signs of illness were slight or absent. No animal died from Q fever. There were no striking temperature reactions; only three animals (two *R. conatus* and one *Hydromys*) had a significant fever, and then for one day only. Variations in weight were too irregular for comment. At necropsy the only naked-eye abnormality was occasional slight enlargement of the spleen.

This study shows that *R. burneti* has a wide range of potential hosts, and suggests that a number of animals may with the bandicoot provide the natural reservoir of infection. This is supported in the case of *R.c. youngi* and *H. chrysogaster* by evidence from agglutination. That many animals are susceptible brings Q fever into accord with other rickettsioses. For instance, Wolbach (1940) states:

"In contrast to the vector-borne protozoal diseases, the rickettsial diseases are remarkable for the number of possible vectors and the many mammalian species susceptible to each disease. . . . In the case of Rocky Mountain spotted fever, all North American rodents, man, monkeys and dogs are susceptible to more or less severe infections."

SUMMARY.

Nine species of bush animals apart from the bandicoot were tested for susceptibility to Q fever. These comprised seven rodents: *Rattus assimilis*, *R. conatus*, *R. culmorum* (two subspecies), *R. lutreolus*, *Hydromys chrysogaster*, *Melomys littoralis* and *Thetomys gracilicaudatus*, and two marsupials: *Trichosurus vulpecula* and *Ærypprymnus rufescens*. All proved susceptible.

The infection in all cases was mild, and often inapparent.

The potential host range of *R. burneti* is therefore wide. As most of the species tested exist in the area where Q fever is endemic, it is likely that some of them become infected naturally, and together with the bandicoot provide the reservoir of infection.

The laboratory rabbit is mildly susceptible to Q fever.

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REFERENCES.

Derrick, E. H. (1937): Med. J. Austral., 2, p. 281.
Freeman, M. *et al.* (1940): Austral. J. exp Biol., 18, p. 193.
Troughton, E. LeG. (1939): Records of the Australian Museum, 20, p. 278.
Wolbach, S. B. (1940): In Virus and Rickettsial Diseases, Harvard University Press, p. 792.